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**THE DEVELOPMENT AND EVALUATION OF A TIME-RESOLVED  
FLUOROIMMUNOASSAY FOR LIPOPROTEIN (a) IN HUMAN SERUM**

A thesis submitted to the Open University for the M.Phil. Degree.

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## **PREFACE**

All experiments and results described in this thesis are my own work, excepting the production and identification of anti-Lp(a) monoclonal antibodies which was performed in collaboration with Drs Wang, Wooley and Byrne in the Department of Clinical Biochemistry, Addenbrooke's Hospital. The work was carried out between April 1992 and July 1994.

This thesis does not exceed 40,000 words in length and has not been submitted for a degree, diploma or other qualification at any other University.

**Keith A Burling**

**25 September 1994.**

## SUMMARY

Lipoprotein (a) (Lp(a)) has a complex structure which includes two apolipoproteins, apolipoprotein B (which is also present in other serum lipoproteins) and apolipoprotein (a) (which is unique to Lp(a)). Raised serum concentrations of Lp(a) have been associated with an increased risk of coronary heart disease.

Commercially-available Lp(a) assay systems suffer from poor analytical performance. In this study, cell lines secreting anti-Lp(a) antibodies are identified using a series of immunoassay-based screening tests. Monoclonal antibodies developed from these cells lines are used (in conjunction with commercially-available polyclonal anti-Lp(a) antibodies) to develop a two-site time-resolved fluoroimmunoassay for Lp(a) in human serum. This assay format requires one antibody to be immobilised onto the surface of a microtitre plate and a second antibody to be labelled with an element from the lanthanide series (europium) which, under certain reaction conditions, is fluorescent. Protocols for the labelling of polyclonal and monoclonal antibodies with europium are presented in this thesis.

Various combinations of anti-Lp(a) antibodies are used in assay development. The combination found to give the best assay performance with the minimum of interference from other biomolecules was:- a monoclonal anti-apo (a) capture antibody with a europium-labelled polyclonal anti-apo B detection antibody. Data obtained during the optimisation of antibody concentrations, sample dilution and reaction times is also presented. In the optimised assay, 200 $\mu$ l of a 1:1000 dilution of serum is incubated with the capture antibody for three hours. Following a wash step, immune complexes are detected fluorimetrically after incubation with a europium-labelled second antibody for two hours.

The performance of the assay is compared to that of commercially-available kit assays. The time-resolved fluorescence assay is shown to be more precise and to have a much wider working range (1-1500 mg/L) than the commercial assays. However, data from analysis of samples distributed as part of an external quality control scheme is used to demonstrate the wide inter-assay variability of Lp(a) measurements.

Measured Lp(a) concentrations in a series of human serum samples demonstrate the highly skewed distribution previously reported in adult Caucasian populations. No significant differences are observed in the mean serum Lp(a) concentrations of males and females and no correlation could be made between age and serum Lp(a) concentration in the adult population studied. In a limited study, a wide day-to-day percentage variation in serum Lp(a) concentration is demonstrated in one subject.

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# **CHAPTER 1**

## **INTRODUCTION**

Lipids are present in human plasma in four distinct forms :-

(i) *Fatty acids* - are straight-chained compounds of varying length which may be saturated (containing no carbon-carbon double bonds) or unsaturated (containing one or more C-C double bonds). Free, or non-esterified fatty acids (FFA or NEFA) which are carried in the blood bound mainly to albumin, are an immediately-available source of energy. Fatty acids may also be esterified with glycerol to form glycerides.

(ii) *Triglycerides* - consist of glycerol esterified with three fatty acids.

(iii) *Phospholipids* - are complex structures containing triglycerides together with hydrophilic phosphate groups and other hydrophobic moieties such as nitrogenous bases.

(iv) *Cholesterol* - has a sterol ring structure which is the precursor of various steroids molecules. Approximately two-thirds of serum cholesterol is esterified as cholesterol esters.

Lipids are hydrophobic and are therefore transported in body fluids as soluble lipid/protein complexes called lipoproteins. Lipoproteins consist of a core of insoluble (non-polar) triglyceride and cholesterol esters enclosed by soluble (polar) proteins, phospholipids and free cholesterol. The protein components may be divided into five principal groups known as apoproteins A, B, C, D and E.

Lipoproteins vary in size and composition, and are commonly classified by density using ultracentrifugation. There are four main classes: high density lipoprotein (HDL) and low density lipoprotein (LDL) transport cholesterol, whereas very low density lipoprotein (VLDL) and chylomicrons transport triglycerides. A fifth class, IDL (intermediate density lipoprotein), usually occurs as a transient intermediate during the metabolism of VLDL to LDL <sup>(1)</sup>.

Raised concentrations of plasma lipids may result in the accumulation of lipid in tissues, particularly in the endothelium of arterial walls, leading to cell damage <sup>(1)</sup>. However, there is considerable evidence that, whereas increased concentrations of LDL are associated with cardiovascular disease, an elevated concentration of HDL

protects against cardiovascular disease. This is presumably due to its role of transporting cholesterol from peripheral tissues for excretion <sup>(1)</sup>.

Lipoprotein (a) (Lp(a)) is a plasma lipoprotein that has characteristics similar to LDL. Both contain apolipoprotein B<sub>100</sub> (apo B) but in Lp(a) (unlike LDL) the apo B is linked to apolipoprotein (a) (apo (a)) by disulphide bridges. Interest in lipoprotein (a) has grown enormously over the last decade as elevated plasma concentrations have been linked to an increased risk of coronary heart disease <sup>(1)</sup>.

### **1.1 The discovery of Lipoprotein (a).**

Lp(a) was first described by Berg in 1963 <sup>(2)</sup> who attempted to demonstrate antigenic differences in the human lipoproteins migrating in the beta region <sup>(3)</sup> of serum protein electrophoretic strips by immunising rabbits with a human LDL fraction. The immune sera produced when absorbed with LDL present in test serum samples, detected a serum antigen in some but not in all individuals. Positive reactors were designated, Lp(a+), and negative reactors, Lp(a-). Much of the early characterisation of the protein was performed by Berg in the mid-1960 s: ultracentrifugation showed that Lp(a) antigen was present in the lipoprotein density class 1019-1063 g/L <sup>(4)</sup>. Family studies indicated that the presence of the Lp(a) antigen was genetically determined, being found in 30-40% of subjects studied in a variety of populations <sup>(5)</sup>.

At this time, Lp(a) was considered to be a genetic variant of LDL <sup>(5)</sup>. However, chemical studies performed in the early 1970 s disproved this hypothesis: Ehnholm, Simons and Garoff demonstrated that although Lp(a) had a lipid composition similar to LDL, it contains significantly more carbohydrate than LDL and has pre-beta electrophoretic mobility <sup>(6)</sup>. When serum was subjected to ultracentrifugation, the low density pre-beta lipoproteins "floated" but Lp(a) "sunk". Lp(a) was therefore referred to as "sinking pre-beta lipoprotein" <sup>(7)</sup>. Lp(a) has also been found in other ultracentrifugation fractions: Utermann and his co-workers

detected Lp(a) in the 1060-1120 g/L density fraction <sup>(8,9)</sup> while studies of the narrow density class, 1050-1060 g/L, showed the presence of HDL, LDL and Lp(a) <sup>(10)</sup>.

In the early 1980s the structure of the Lp(a) lipoprotein was published independently by three groups of workers <sup>(11-13)</sup>: Lp(a) is a complex macromolecular structure containing a high molecular weight glycoprotein termed apolipoprotein (a) (apo(a)) linked to apo B by disulphide bonds. The structure of Lp(a) will be described in more detail later in this introduction.

Positive association between raised plasma concentrations of Lp(a) and increased risk of myocardial infarction was first described by Renninger *et al* <sup>(14)</sup> in 1965. Furthermore, increased concentrations of slow pre-beta lipoprotein were demonstrated in electrophoresis of serum from patients with atherosclerotic disease <sup>(15)</sup>. This protein was subsequently demonstrated to be Lp(a). Following these observations there has been much interest in Lp(a) as an independent marker of coronary heart disease <sup>(16)</sup>, and many papers have been published on the subject. However, quantitative assay of Lp(a) has yet to find a place in the test repertoire offered by most hospital laboratories in Great Britain. This may be due to deficiencies in the analytical technique, difficulties in interpretation of results, or perhaps more importantly, because no effective treatment for lowering serum Lp(a) concentrations is yet available.

## **1.2 Inheritance of Lipoprotein (a).**

The inheritance of lipoprotein (a) was first investigated by Berg and his co-workers in 1967. Analysis of serum from the members of 500 Austrian families demonstrated that Lp(a) inheritance was compatible with a simple dominant Mendelian trait under the control of two alleles termed Lp<sup>a</sup> and Lp<sup>0</sup> <sup>(17)</sup>. However, this interpretation was challenged by two groups in the early 1970s who noticed that the distribution of Lp(a) in similar sized Caucasian populations was continuous but highly skewed <sup>(18,19)</sup>, with most subjects falling in the low concentration range. This was not compatible with Berg's theory. Extended family studies by Sing *et al* <sup>(20)</sup> concluded

that one major gene with a polygenic background determined plasma Lp(a) concentrations whereas Hasstedt *et al* <sup>(21)</sup>, Hasstedt and Williams <sup>(22)</sup> and Morton *et al* <sup>(23)</sup> have suggested that Lp(a) concentrations are controlled by two or three alleles that determine overlapping distributions. The nature of this postulated locus remains undefined, and to this day the mode of Lp(a) inheritance is not completely understood. The apo (a) gene and protein has also been detected in old world primates <sup>(24)</sup> and hedgehogs <sup>(25)</sup> but not in other animals.

### 1.3 Physicochemical structure of Lp(a).

The structure of Lp(a) has been extensively investigated. Lp(a) consists of two apolipoproteins, apo (a) and apo B, linked by a disulphide bridge (or bridges). Apo (a) can be quantitatively removed from Lp(a) by ultracentrifugation under reducing conditions. The resulting apo(a)-deficient lipoprotein is found in the same fraction as LDL while the essentially lipid-free apo (a) is recovered as the lower fraction on ultracentrifugation <sup>(26)</sup>. Fless and his co-workers <sup>(27)</sup> have shown that apo (a)-deficient Lp(a) is a larger particle than LDL, and contains comparatively more triglycerides. The density of intact Lp(a) is greater than LDL, while apo (a)-deficient Lp(a) is the same as LDL <sup>(28)</sup>. It may therefore be concluded that the major differences between the physicochemical properties of Lp(a) and those of LDL result from the presence of apo(a).

Little was known about the structure of Lp(a) until the mid 1980 s when studies by Fless and Scanu <sup>(29)</sup> and Gaubatz *et al* <sup>(30)</sup> established that apo (a) was a glycoprotein with a high degree of polymorphism between individuals resulting in molecular weights higher than, lower than or equal to the molecular weight of apo B (which is approximately 500 kDa). Hence Lp(a) varies in size between 400 and 838 kDa <sup>(30,31)</sup>.

Kringles (derived from the old Norse *kringla*) are ring shaped structures with 78 - 82 amino acids, stabilised by three internal disulphide bonds which are present in various plasma proteins involved in coagulation and fibrinolysis <sup>(33,34)</sup>. Amino acid

sequence data demonstrated that apo (a) is made predominantly out of kringle structures, containing one kringle 5 domain and between 5 and 37 kringle 4 domains. In addition apo (a) also contains a serine protease region which has 94% structural homology with plasminogen but has no enzymatic activity <sup>(35)</sup>. Thus, the size differences observed in apo (a) phenotypes are due primarily to the number of kringle 4 repeat units although some differences in glycosylation of the protein may also contribute <sup>(32)</sup>. Plasminogen also contains the kringle 4 and 5 domains, but kringles 1, 2 and 3 (which are found in plasminogen) are not present in Lp(a). The high degree of homology between plasminogen and apo (a) is exemplified by the cross-reactivity of antibodies raised against each protein <sup>(26, 36)</sup> and by the fact that, like plasminogen, apo (a) is retained by lysine-sepharose chromatography columns <sup>(37)</sup>.

The wide variation in the molecular mass of apo (a) (and hence Lp(a)) results in considerable variation in the number of isoforms (6-23) observed in various human populations <sup>(38, 39)</sup>. Lp(a) isoforms are usually analysed using immunoblotting: Following electrophoresis in a polyacrylamide gel (under reducing conditions) the separated Lp(a) isoforms are blotted onto a nitrocellulose membrane. Lp(a) is labelled with a specific antibody and the resulting immune complexes are detected using a second (anti-species) antibody covalently coupled to an enzyme such as horseradish peroxidase or a radioisotope such as <sup>125</sup>I <sup>(38)</sup>. The separated Lp(a) isoforms may then be classified according to their relative electrophoretic mobility compared to that of apo B and assigned molecular masses by comparison with the migration of marker proteins of known molecular weights <sup>(38)</sup>. Thus the F isoform (400 kDa) has a faster electrophoretic mobility than apo B, the B isoform (460 kDa) has the same mobility as apo B, and the S1, S2, S3 and S4 isoforms (520, 580, 640 and 700 kDa respectively) each have slower mobility than apo B. Whereas each of the aforementioned species migrate as a single band, other isoforms (BS1, BS2, BS3, S1S2, S2S3, S2S4, S3S4) migrate as doublets. The method described by Utermann *et al* <sup>(38)</sup> employs a primary polyclonal rabbit anti-Lp (a) antibody followed by a <sup>125</sup>I-labelled anti-rabbit second antibody. In spite of the sensitivity of this technique (90 ng of



Lp(a), equivalent to a plasma concentration of ~70 mg/L), 121 out of 247 (49%) subjects studied had plasma Lp(a) concentrations below the detection limit. Of the remaining 126 subjects, 14 had a doublet isoform (the most common being S2S3 which was detected in 4 subjects), no subject had the F isoform and only 4 subjects had the B isoform. However, the larger S1 - S4 isoforms were found in 18, 33, 33 and 25 subjects respectively.

There appears to be a significant inverse relationship between the molecular mass of apo (a) isoforms and plasma Lp(a) concentrations <sup>(31)</sup>. Smaller isoforms (which are present at much lower frequency in the population) are associated with high plasma Lp(a) concentrations, while the larger isoforms (which are found at a much higher frequency) are associated with lower plasma Lp(a) concentrations. However, a 100-fold variation in plasma Lp(a) concentration has been observed amongst individuals with the same isoform <sup>(31)</sup>.

#### **1.4 Distribution of Lp(a) in human populations.**

Many studies of Lp(a) distribution in human populations have been performed. Berg's early qualitative assays showed consistent proportions of positive reactors (those exhibiting the Lp(a+) antigen) within various ethnic groups. Thus, around 35% of various white populations, 34% of American blacks and 30% of Greenland eskimos were positive for Lp(a). However, a significantly lower incidence of positive reactors was observed in Labrador Indians (2%) and natives of Easter Island (8%) <sup>(40)</sup>. One of the first quantitative population studies was performed in Sweden <sup>(41)</sup> where a highly skewed distribution of serum Lp(a) concentrations is found. Two-and-a-half percent of the population had Lp(a) concentrations above 480 mg/L, the value was chosen as their upper reference value for human serum Lp(a) concentrations. This distribution pattern was reproduced in studies involving adult Japanese males <sup>(44)</sup> and other Caucasian populations <sup>(38,43-45)</sup>, all of which showed the same skewed distribution with a median concentration of ~120-150 mg/L. However, some interesting variations have been observed. A study by Viikari *et al* in

Finland in 1990 <sup>(43)</sup> quantitated Lp(a) in serum from 2465 Finnish children and young adults between the ages of 9 and 24. The usual skewed distribution pattern and median concentration were observed and there was no difference in serum Lp(a) concentration between boys and girls, or between age groups. However, when the population was divided according to region, it was apparent that an excess of low values occurred in subjects from West Finland. Interestingly, a higher incidence of coronary heart disease was seen in East Finland. Guyton *et al* described a population of black American men and women <sup>(46)</sup> which showed a bell-shaped distribution of serum Lp(a) concentration with the mean value double that of the white American population. The population of the Indian subcontinent have an Lp(a) distribution intermediate between other Caucasians and blacks <sup>(47)</sup>, while the Lp(a) distribution in the Chinese population is more skewed than in other populations <sup>(31)</sup> (with a greater proportion of low values). These findings may suggest that serum Lp(a) concentrations are race-dependent <sup>(48)</sup>.

One study <sup>(49)</sup> measured serum Lp(a) during the first year of life. Concentrations were shown increased from a mean value of 40 mg/L at birth up to 78 mg/L at 3 months and 108 mg/L at 6 months of age. The mean adult concentration was quoted at 140 mg/L. In contrast to adults, no significant differences in Lp(a) concentrations have been demonstrated between black and white neonates <sup>(50)</sup>.

### **1.5 Serum Lp(a) concentrations in health and disease.**

A positive correlation between high serum Lp(a) concentrations and an increased incidence of atherosclerotic disease has been thoroughly documented <sup>(51-83)</sup>. The concentration of Lp(a) considered to be abnormal was reassessed by Dahlen *et al* in 1986 <sup>(60)</sup>. He studied a group of 307 American males and females and found that a Lp(a) concentration of 300 mg/L corresponded with the 70th percentile of the serum Lp(a) distribution. This concentration was found to be a threshold, below which Lp(a) seemed to have no effect on coronary artery stenosis risk scores in males or females below the age of 56.

The regulation of plasma Lp(a) concentrations is not fully understood. The major site of Lp(a) synthesis appears to be in the liver since liver transplant recipients adopt the apo (a) isoform of the donor <sup>(84)</sup>. Although Lp(a) and LDL both contain apo B, they are metabolically distinct lipoproteins and, unlike LDL, Lp(a) does not appear to be a by-product of VLDL metabolism <sup>(85)</sup>. Lp(a) can be associated with triglyceride-rich particles found in individuals with hypertriglyceridaemia <sup>(86)</sup>, but cholesterol feeding (which can substantially increase serum concentrations of apo B and LDL) has little effect on serum Lp(a) concentrations <sup>(87)</sup>.

The mechanism of Lp(a) degradation is uncertain. Studies *in vivo* suggest that the LDL receptor plays a role in the catabolism of Lp(a) <sup>(88-90)</sup>, although other studies suggest that this is not a major clearance route <sup>(91,92)</sup>. Subjects with an apo B mutation that abolishes binding to the LDL receptor do not have increased concentrations of Lp(a) <sup>(31)</sup>. However, subjects with a defect in the LDL receptor itself do have elevated serum Lp(a) concentrations <sup>(93)</sup>. A study by Hofmann *et al* <sup>(94)</sup> showed that Lp(a) binds to the LDL receptor with an affinity similar to that of LDL, and that clearance of human Lp(a) is markedly increased when it is administered to transgenic mice that overexpress human LDL receptors.

Structural similarities between Lp(a) and plasminogen are thought to be a possible cause of the increased risk of atherosclerosis in subjects with raised serum Lp(a) concentration. Tissue plasminogen activator acts on plasminogen to release plasmin, a proteolytic enzyme responsible for the breakdown of fibrin clots. Lp(a) might interfere with plasmin generation by (i) competing with plasminogen for cell surface receptors <sup>(95-97)</sup>, (ii) by inhibiting the conversion of inactive plasminogen to active plasmin <sup>(98,99)</sup> or, (iii) by competing with plasmin for binding sites on fibrin <sup>(99,100)</sup>. It has been shown that the kringle 4 domain in Lp(a) avidly binds to fibrin *in vitro* <sup>(100)</sup>. Fibrin is abundant in early atheromatous plaques <sup>(101)</sup> and there is strong evidence that serum Lp(a) binds to this fibrin (in preference to other apo B-containing serum proteins such as LDL) <sup>(101)</sup>. The Lp(a) may then be modified by oxidation and taken up by macrophages, thus contributing to foam cell formation within plaques

(102). *In vitro* studies have shown that Lp(a) competes in equimolar concentrations with plasminogen for binding to plasminogen receptors on vascular endothelial cells (96). However, using immunochemistry, Lp(a) could not be detected on plasminogen receptors on colon or breast carcinoma cells (103). Although Lp(a) was thought to have no enzymic activity (80), it has been shown to bind to and cleave (albeit slowly) the extracellular matrix protein, fibronectin, *in vivo* (104).

Several groups have described variations in serum Lp(a) concentrations in acquired medical conditions. For example, in acute renal disorders associated with proteinuria, serum Lp(a) concentrations are two-fold higher than during remission (105,106). Haemodialysis had no effect on serum Lp(a) concentrations in a group of patients with chronic renal failure who also had a raised serum Lp(a) concentration (107,108). Elevated Lp(a) concentrations have also been observed in type 1 (insulin-dependent) diabetics with microvascular disease (109-112) and in type 2 (non insulin-dependent) diabetics with poor glycaemic control (113). However, improved glycaemic control was associated with a lowering of Lp(a) concentrations in the latter group (112-114). One study suggested that all diabetics show a tendency towards higher serum Lp(a) concentrations (115).

Originally Lp(a) was believed to be an acute phase protein (116), serum concentrations of which increase during various acute inflammatory conditions. However, recent studies suggest that this is not the case. For example, Slunga *et al* (117) showed that Lp(a) concentrations rose only very slightly following myocardial infarction and that this rise did not correlate with rises in the concentrations of other acute phase proteins such as orosomucoid, C-reactive protein and alpha-1-antitrypsin. However, the rise in Lp(a) concentration did correlate with elevations of other plasma lipids. These findings were supported by similar observations in a group of patients with rheumatoid arthritis (118).

The concept of an hepatic site of Lp(a) synthesis is further supported by two groups. One group showed that serum Lp(a) concentrations were very low in patients with alcoholic cirrhosis (119), while the other study involved 18 patients with biliary

cirrhosis or chronic active hepatitis <sup>(120)</sup>, 14 of these subjects were found to have undetectable serum concentrations of Lp(a). However, the other four had raised serum Lp(a) concentrations, suggesting an additional extra-hepatic site for Lp(a) synthesis. An interesting study by Kervinen *et al* <sup>(121)</sup> showed a rapid increase in plasma Lp(a) concentration in some alcoholic men four days after ethanol withdrawal. The most dramatic increase in concentration was approximately two and a half times the basal level, although some subjects showed little or no increase. The immunosuppressive drug, cyclosporin, was associated with an elevation in serum Lp(a) concentration in a group of renal transplant recipients when compared to a control group receiving alternative immunosuppressive therapy <sup>(122)</sup>. Possible nephrotoxic effects of cyclosporin were not considered to be responsible for the increase in Lp(a) since renal function was similar in each group of patients.

Diet appears to have little effect on plasma Lp(a) concentration <sup>(7,29,30)</sup>. Lipid lowering drugs such as hydroxymethylglutaryl coenzyme A reductase inhibitors (simvastatin, pravastatin), bile acid sequestrants (cholestyramine), fibric acid derivatives (clofibrate, gemfibrozil) and the antioxidant, probucol, which have a significant lowering effect on plasma LDL concentrations have no effect on plasma Lp(a) concentration <sup>(31,123-128)</sup>. However, Lp(a) concentrations can be decreased by treatment with nicotinic acid (which can be used to treat peripheral vascular disease) or a combination of nicotinic acid and the aminoglycoside antibiotic drug, neomycin (which inhibits the transport of non-esterified fatty acids to the liver). However, the hepatotoxicity of these drugs precludes their use in long-term treatment <sup>(127,128)</sup>. N-acetyl cysteine (which is thought to reduce the formation of disulphide bonds within Lp(a)), has been reported to successfully lower serum Lp(a) concentration in two patients <sup>(129)</sup>, although other workers were unable to reproduce these results <sup>(130)</sup>. The anabolic steroid, stanozolol, <sup>(131)</sup> and androgenic progestogens (a component of hormone replacement therapy) also reduce plasma Lp(a) concentrations <sup>(132)</sup>. This action of progestogens may, to some extent counterbalance the undesirable increases in the plasma concentration of other lipids observed with these drugs <sup>(133)</sup>. Hormonal

regulation of serum Lp(a) concentration has also been observed during the treatment of prostatic carcinoma: whereas Lp(a) concentrations rose slightly in patients treated by orchidectomy, Lp(a) concentrations decreased in those undergoing oestrogen treatment <sup>(134)</sup>. One group has described a rise in serum Lp(a) concentrations up to the 25th week of normal pregnancy, with a rapid return to normal post-partum which may be related to the hormonal changes of pregnancy <sup>(135)</sup>. Fish oils, which decrease the secretion of VLDL, also appear to lower plasma Lp(a) concentrations <sup>(123,137,138)</sup> but the effectiveness of fish oils as an Lp(a)-lowering treatment has yet to be confirmed. Cigarette smoking, which is a recognised risk factor for coronary artery disease, has no significant effect on serum Lp(a) concentration <sup>(139-140)</sup>. Since there is no suitable safe means of lowering plasma Lp(a) concentration it may be that the treatment of individuals with a high risk of coronary artery disease should be based on reducing other risk factors <sup>(76)</sup>.

## **1.6 Qualitative and quantitative measurement of Lp(a).**

Early qualitative methods for the detection of Lp(a) employed gel diffusion under non-denaturing conditions in agarose or starch gels <sup>(7,9)</sup>. Immune complexes were visualised using lipophilic stains such as Sudan black or Oil red. However, the subsequent availability of specific antibodies to Lp(a) has allowed the development of quantitative assays. The first generation of quantitative assays (radial immunodiffusion (RID) <sup>(141)</sup> and electroimmunodiffusion (EID) <sup>(142)</sup>) employed precipitation of antigen-antibody complexes within gels <sup>(19,143-145)</sup>. However, RID suffers from several problems: the technique is insufficiently sensitive to measure Lp(a) in all human serum samples and, more importantly, size differences between the Lp(a) particles influences their mobility in the gel <sup>(146)</sup>. Most studies performed in the 1970 s, which correlated raised concentrations of Lp(a) with an increased risk of cardiovascular disease, used EID assays. The technique had adequate sensitivity and reproducibility but was very labour-intensive and not suitable for automation.

Radioimmunoassay (RIA) <sup>(147)</sup> for Lp(a) was developed in the late 1970 s <sup>(86)</sup>.

The method is sensitive and specific but uses radioisotopic reagents with limited shelf-life. Immunoradiometric assays (IRMA) <sup>(148,149)</sup> were developed later <sup>(150)</sup>. These two-site ("sandwich") assays employ a primary antibody adsorbed onto a solid phase, and a second antibody labelled with <sup>125</sup>I. The analytical performance of these assays is adequate and large batches of samples may easily be assayed using automated equipment.

Immunoturbidimetric and immunonephelometric assays have also been used to quantitate Lp(a) <sup>(151,152)</sup>. Each of these techniques is based on the precipitation reaction between Lp(a) and an anti-Lp(a) antibody in solution. In the turbidimetric assay, the formation of antigen-antibody complexes prevents monochromatic light passing through a cuvette, the amount of light blocked being proportional to the Lp(a) concentration. In nephelometric assays, light scattered by the antigen-antibody complex is measured at right angles to the incident light. Although each of these methods are easy to automate, they suffer from several drawbacks. For example, they are very susceptible to interference from other biomolecules (eg high concentrations of triglycerides <sup>(153)</sup>). In addition, repeated freezing and thawing of serum samples appears to decrease measured Lp(a) concentrations to a greater extent than is observed using alternative analytical methods <sup>(154)</sup>. Although nephelometric methods are highly sensitive and do not require sample dilution they are strongly influenced by variation in the size of the Lp(a) isoforms which have associated variations in their light scattering properties <sup>(153)</sup>. However, they are simple and quick to perform, and large numbers of samples may be analysed using automated equipment. However, analytical results appear to be less reproducible than those obtained using IRMA assays <sup>(152)</sup>.

The wide availability of microtitre plate assays in the early 1980 s led to the development of a number of enzyme-linked immunosorbent assays (ELISA) for Lp(a) <sup>(155-166)</sup>. These assays use an anti-Lp(a) antibody adsorbed onto plastic microtitre plates to capture Lp(a) in the sample (which has previously been diluted between 500- and 2000- fold). A second anti-Lp(a) antibody, conjugated to an enzyme such as

horseradish peroxidase, is used to detect the amount of Lp(a) bound to the capture antibody. The immobilised enzyme subsequently catalyses the production of a coloured product from an added substrate. The intensity of the colour produced (which is measured photometrically) is proportional to the Lp(a) concentration in the sample. ELISA assays are easy to perform, easy to automate and (with good assay technique) highly reproducible. However, they suffer from a limited working range (~10-800 mg/L) which means a minority of subjects have apparently unmeasurable serum concentrations of Lp(a). In 1992 ten out of fourteen participants in a national quality assurance scheme run from Manchester, UK <sup>(154)</sup> used one of three commercially available ELISA kits. The major advantages of using these kits were that no in-house reagent production was required, the reagents had a long shelf life and there were no major safety hazards. However, the cost of commercial ELISA kits is quite high, typically around £200 for a kit sufficient to analyse 36 serum samples in duplicate.

An interesting alternative approach to the measurement of Lp(a) was made by Lou *et al* in 1992 <sup>(167)</sup>, who developed a semi-quantitative immunochromatographic method. This assay uses a nitrocellulose membrane strip coated in each of four regions by an anti-Lp(a) monoclonal antibody. Purified, dried Lp(a), coated with colloidal selenium (rusty red in colour), is immobilised on a glass fibre pad at one end of the strip. The dried Lp(a) is rehydrated by the addition of 25µl of plasma (or 50µl of whole blood : an anti-erythrocyte antiserum is also impregnated in the glass fibre pad to immobilise red cells). The mixture of sample Lp(a) and selenium-coated Lp(a) diffuses through the nitrocellulose membrane strip and competes for binding in the regions coated with anti-Lp(a) antibody. If the Lp(a) concentration in the serum sample is low, the binding capacity of the first antibody region is sufficient to bind all the serum- and selenium-coated Lp(a). Hence, only the first antibody coated region would be coloured. In samples with very high Lp(a) concentrations the binding capacity of all four antibody-coated regions would be saturated with Lp(a), and each



region would therefore be coloured red. Analytical time is only ~10 minutes for each sample, and the results are highly reproducible and agree well with ELISA assays.

### 1.7 Time-resolved fluorescence immunoassay (DELFI<sup>®</sup>A) for Lp(a).

Dissociation Enhanced Lanthanide FluoroImmunoAssay (DELFI<sup>®</sup>A) (also known as time-resolved fluorescence immunoassay) has been used since the early 1980 s as an alternative to radioisotopic and enzyme-labelled immunoassays. Most of the development of DELFI<sup>®</sup>A was performed in Scandinavia by Hemmilä and his co-workers <sup>(168-173)</sup>. A large number of DELFI<sup>®</sup>A kits are now commercially available for the measurement of peptide hormones such as thyroid-stimulating hormone, lutenising hormone (LH), follicle-stimulating hormone (FSH) and human chorionic gonadotrophin (hCG), the steroid, cortisol, proteins such as prostate specific antigen and alphafoetoprotein and drugs such as digoxin <sup>(174-179)</sup>. More kits for other analytes are becoming available each year.

The sensitivity of conventional fluorescence immunoassays is severely limited by unavoidably high background fluorescence due to the scattering of light by solvents, solutes and particles in solution and from the inherent fluorescence of biomolecules including proteins and NADH. The fluorescent decay times of these compounds is in the region of 10 nsec. Time-resolved fluorescence overcomes the problem of background fluorescence by using a fluorophore with a decay time in excess of 500 *microseconds* ( $\mu$ sec). Hence, a fluorescent signal measured at a time greater than 10 nsec (and less than 1 millisecond) will be free from interference by background fluorescence. The elimination of background fluorescence together with a fluorimeter capable of measuring fluorescent counts in excess of 1 million/sec has allowed the development of highly sensitive assays with a working range spanning up to 5 orders of magnitude. Time-resolved fluorescence immunoassays employ reagents which are stable for up to one year and have none of the safety hazards associated with radioimmunoassays <sup>(180)</sup>.

Most commercial DELFIA® assays are in the form of a two-site immunoassay with a primary monoclonal "capture" antibody which is coated onto a microtitre plate and a secondary monoclonal antibody labelled with a fluorophore such as europium. Europium (Eu), with an atomic weight of 152, is a member of the lanthanide group of elements which also includes samarium, terbium and dysprosium. It is similar in atomic size to iodine (atomic weight 125) which is commonly used as a label in radioimmunoassays, although, in contrast to iodine, europium cannot be directly covalently coupled to a protein. However, europium forms a stable complex with the chelating agent, N1-(p-isothiocyanatobenzyl)diethylenetriamine-N<sup>1</sup>N<sup>2</sup>N<sup>3</sup>-tetraacetic acid (DTTA). This complex contains an isothiocyanate group which reacts with free amino groups in proteins to form a stable covalent thiourea bond. Using Eu/DTTA chelates individual antibody molecules may be labelled with up to 10-20 atoms of europium.

The Eu/DTTA chelate supplied in the kit marketed by Wallac at a cost of around £150 is sufficient to label enough antibody for at least one hundred 96-well microtitre plate assays. Highly purified bovine serum albumin may be added to a final concentration of 0.1% (w/v) to aid stability and the labelled antibody is stored at +4 °C. When stored correctly, the labelled protein is stable for at least one year.

The labelled antibody itself is non-fluorescent. However, after it has bound to the 'captured' antigen (immobilised onto the microtitre plate), europium is dissociated from the secondary antibody by the addition of enhancement solution (a buffer containing a beta diketone, 2-naphthoyl-trifluoroacetate at pH 2-3; a detergent, Triton X-100; and a fatty acid derivative, trioctylphosphine oxide). In solution, the europium ions form highly fluorescent chelates stabilised inside micelles. Lanthanide chelates have a long fluorescence decay time, a very large Stokes shift (i.e. the difference between the excitation wavelength (340 nm) and the emission wavelength (613 nm)), a sharp emission peak and a high fluorescence intensity<sup>(180)</sup>. In addition, the chelates are very stable and, if evaporation is avoided, solutions may be left in microtitre plates for up to 24 hours before fluorescence measurements are made. Dual-labelled diagnostic

kits are now available in which two analytes may be measured simultaneously using the same microtitre plate (eg LH & FSH). One detection antibody is labelled with europium and the other is labelled with samarium. Since europium and samarium have significantly different emission wavelengths (Eu = 613 nm, Sm = 643 nm) and different fluorescent lifetimes (Eu 730  $\mu$ sec and Sm 50  $\mu$ sec), the fluorimeter can easily distinguish between the two. This produces assays with high sensitivity and specificity for each analyte. In theory, it should be possible to measure four different analytes in a single microtitre plate using four lanthanide labels, although this has yet to be achieved.

The availability of the Wallac labelling kit brought DELFIA® assays into the research environment. The labelling of antibodies and other proteins is very simple: 1 mg of the antibody to be labelled is added to 0.2 mg of europium-DTTA chelate and incubated overnight. Labelled antibody is separated from free label using gel filtration chromatography. Labelling yield (the number of europium atoms on each antibody) may then be estimated by calculating the europium and protein concentrations of the labelled antibody solution. The former may be estimated by comparison with a europium solution of known molarity and protein concentration may be estimated using the method described by Lowry <sup>(181)</sup> or by measuring absorbance at 280 nm <sup>(182)</sup>. Typical yield is around 10 europium atoms/antibody. Labelling yield can be increased by varying the incubation time, buffer pH and reaction temperatures: a longer incubation time, higher buffer pH and higher temperature each increase europium incorporation. However, extremes of these parameters may result in protein denaturation, especially when monoclonal antibodies are employed. A yield >3, but <20 europium atoms/antibody results in optimal assay performance.

Time-resolved fluorescence immunoassays have been developed for use in many fields of biomedical science. For example, applications have been found in microbiology, biochemistry and immunology, and a method has also been described for quantifying DNA <sup>(183-196)</sup>. Indeed, it may be that use of time-resolved fluorescence

immunoassays will become as widespread in the 1990 s as radioimmunoassays were in the 1970 s.

### **1.8 Interassay variability for Lp(a).**

The wide range of methods employed in the measurement of Lp(a) has given as much as 2-3 fold variation in the results obtained from patient samples. This was demonstrated by analysing results obtained from samples sent out by the external quality control scheme based in Manchester, UK. <sup>(154)</sup> to laboratories around the country for Lp(a) measurement. Laboratories participating in the scheme used one of five different methods, three were ELISA kits, one an IRMA kit and the other an immunoturbidimetric assay. The differences in Lp(a) results obtained for any particular sample could be attributed to one of two major variations in assay design, namely the antibodies used and the standardisation material employed.

The first Lp(a) assays to be developed employed polyclonal antibodies. To eliminate cross-reactivity with apo B, antisera were pre-absorbed with LDL <sup>(33)</sup>. However, the high degree of homology between apo (a) and plasminogen also caused cross-reactivity problems <sup>(197-201)</sup>. One approach to resolving these problems was the development of a two-site assay (e.g. an ELISA) using an anti-apo (a) antibody as the "capture" antibody and an anti-apo B antibody to detect immune complexes <sup>(156,158,160,162)</sup>. Alternatively two anti-apo (a) monoclonal antibodies directed against different epitopes may be employed <sup>(155,160,161)</sup>.

A document with specific recommendations for the generation and selection of monoclonal antibodies for the measurement of apolipoproteins AI and B was published in 1990 by the International Federation of Clinical Chemistry (IFCC) <sup>(202)</sup>. It has been suggested that these recommendations may also be applied to anti-Lp(a) monoclonal antibodies <sup>(33)</sup>. Since it is important that antibodies should react consistently with all Lp(a) isoforms, selected antibodies should be directed against an epitope located on the

kringle 5 or protease domains, one copy of each being present in each isoform. However, an antibody directed against an epitope on the kringle 4 domain would give a variable analytical performance depending on the number of domains present in a particular isoform <sup>(33)</sup>. For this reason, the use of an anti-kringle 4 antibody as a detection antibody should be avoided <sup>(33)</sup>. However, it may be acceptable to use an anti-kringle 4 antibody to capture Lp(a), providing it reacts with all isoforms identically. The IFCC document states that manufacturers should clearly indicate the immunochemical characteristics of the monoclonal antibodies marketed for use in apolipoprotein immunoassays. Unfortunately, however, this has yet to be achieved.

The calibration of Lp(a) assays was, and still is, a major problem <sup>(203)</sup>. Assays with an anti-apo (a) capture antibody and an anti-apo B detection antibody ((a)/B assay) permit the expression of results on a molar basis since each molecule of Lp(a) contains only one apo B subunit. However, the calibration of assays using two anti-apo (a) antibodies ((a)/(a) assay) is usually in mass units. Commercial assays, using either combinations of antibodies (i.e. (a)/B or (a)/(a)) are generally calibrated in mg/L (or mg/dl).

Accuracy of Lp(a) immunoassays is highly dependant on the availability of a primary Lp(a) standard of known concentration, which may be employed to assign values to a reference material which, in turn, may be used to assign values to calibrators used in the assay itself <sup>(33)</sup>. Delipidated Lp(a) undergoes extreme self-association and irreversible aggregation and is therefore unsuitable as a primary standard <sup>(33)</sup>. Purified Lp(a) has been used as a calibrant, but no consensus has been reached regarding its preparation. Lp(a) may be purified from the 1050-1120 g/L density fraction of plasma using ultracentrifugation followed by gel filtration chromatography <sup>(8,19)</sup>. However, the yield from this method is low (15-20%). Albers *et al* purified Lp(a) from the 1060-1090 g/L HDL density fraction of plasma <sup>(7,19,87)</sup>; however, this preparation was not considered to be representative of total plasma Lp(a). Lp(a) has also been purified from plasma by using anti-apo(a) affinity chromatography columns <sup>(197)</sup>.

An alternative approach to the standardisation of Lp(a) assays is to produce a primary standard of purified apo (a). Apo (a) may be purified relatively easily from Lp(a) by the reduction of disulphide bonds followed by centrifugation to remove lipids and apo B <sup>(13)</sup>. Alternatively, apo (a) has been synthesised using recombinant DNA technology <sup>(33)</sup>. However, apo (a) calibrants suffer from several drawbacks; the most important being that they cannot be used in assays utilising anti-apo B detection antibodies. In addition, monoclonal antibodies may react unpredictably in some assays systems: for example, those raised against purified (reduced) apo (a) <sup>(201)</sup> appear to react poorly with intact (non-reduced) Lp(a) particles while those raised against intact (non-reduced) Lp(a) react poorly with (reduced) apo (a) <sup>(204)</sup>. This suggests that some apo (a) epitopes are destroyed under reducing conditions <sup>(33)</sup>. If this is the case, recombinant apo (a) (containing intact disulphide bonds) is more likely to share the epitopes found on native Lp(a) than of reduced apo (a) <sup>(33)</sup>.

Accurate determinations of the protein mass of primary standard material cannot overcome the problem of intra-individual variations in Lp(a) particle composition, size and hydrated density. In particular, assays using two anti-apo (a) antibodies make no correction for differences in the mass of the various Lp(a) isoforms. Since the choice of Lp(a) calibrant remains arbitrary, a consensus is required before direct comparison of results from different assays may be made <sup>(33)</sup>. At present most commercial Lp(a) calibrants consist of lyophilised human plasma from subjects with elevated serum Lp(a) concentration. Calibrants may contain a single isoform of intermediate size or a mixture of several of the isoforms <sup>(33)</sup>. The latter approach may minimise differences resulting from variations in isoform size.

The (a)/B assay format has some advantages: Lp(a) may be quantitated on a molar basis, the quantitation should be independent of isoform size (providing the capture antibody recognises all isoforms equally) and there should be no interference from plasminogen. A recent comparison of Lp(a) measurements <sup>(162)</sup> in serum samples containing each of the main groups of isoforms demonstrated that significantly lower Lp(a) concentrations were obtained with the lower molecular

weight isoforms, F, B and S1, when analysed using an (a)/B assay compared to the (a)/(a) assay. This difference was reversed with the higher molecular weight phenotypes, S2, S3 and S4. The (a)/(a) assay employed in this study was the Macra ELISA kit (Terumo Medical Corp., Elkton, MD 21921, USA) which is currently commercially available only in the USA. The kit contains a monoclonal antibody (1D1) raised against the kringle 4 domain as the solid phase capture antibody, and a peroxidase-conjugated anti-Lp(a) polyclonal antibody as the detection antibody <sup>(205)</sup>. The discrepancies described above may be due to the anti-kringle 4 antibody 'capturing' the different isoforms of apo (a) with different affinities or the polyclonal detection antibody binding to the variable number of kringle 4 domains present in the apo (a) of each isoform.

Many recent publications of quantitative Lp(a) measurements have used ELISA kit assays similar to the one described above. However, it may be impossible to compare results obtained from two groups of workers who use different kits and different standardisation procedures.

### **1.9 *In vitro* stability of Lp(a).**

In addition to antibody and standardisation problems, the stability of Lp(a) *in vitro* has been questioned <sup>(205)</sup>. For any laboratory test to be of clinical use the potential causes of result variability must be understood. Panteghini & Pagani <sup>(205)</sup> suggested that there was a considerable time-dependant biological variation in serum Lp(a) concentration within individuals: Blood samples were collected weekly for five weeks from a group of 4 healthy males and 4 healthy females. Lp(a) quantitation using the Macra ELISA kit gave a mean Lp(a) concentration of 140 mg/L with a mean within-subject variation of 8.5%. This represents a larger biological variation than occurs, for example, with serum total cholesterol. However, the assay used in this study had poor analytical performance; at ~100 mg/L the within-run precision calculated from 20 replicates gave a coefficient of variation of 10.5%, while the between-batch imprecision calculated from 20 replicates gave a coefficient of

variation of 15.7%! This relatively poor performance must have compounded any biological variation.

These authors also examined the effects of sample storage and showed that serum samples were stable for up to 15 days at +4°C and for up to three months at -20°C. Other workers <sup>(87,208,209)</sup> report similar findings although one group suggested that higher molecular weight Lp(a) isoforms undergo considerable cryoprecipitation during storage resulting in loss of immunoreactivity <sup>(13,156,210)</sup>. A study involving samples stored for 6 months at either -20 or -70 °C and then analysed using RID and each of two ELISA assays <sup>(208)</sup> demonstrated no differences in measured Lp(a) concentration using the ELISA assays, but a 46% reduction in measured Lp(a) concentration was apparent using RID. This finding was consistent with concentrations measured in 215 serum samples stored at -40 °C for an average of ten years which were 62% lower by RID <sup>(208)</sup> than by ELISA. Hence, RID appears to be more sensitive to sample deterioration during storage than ELISA.

Recently, it has been reported that lyophilisation of serum consistently results in lower measured Lp(a) concentrations when compared to original fresh serum irrespective of the analytical method employed <sup>(209)</sup>. However, the addition of crystalline sucrose to a final concentration of 0.6 M appears to significantly improve the stability of Lp(a) in lyophilised serum<sup>(210,211)</sup>, although the addition of liquid sucrose (to the same final concentration) was less effective. It was thought that the loss of Lp(a) immunoreactivity following lyophilisation may result from some form of self-aggregation of either (or both) apo B and apo (a) <sup>(211)</sup>.

Median levels of Lp(a) measured in samples from 17 healthy blood donor volunteers indicated no difference between serum samples and plasma anticoagulated with K<sub>2</sub>EDTA <sup>(206)</sup>. No data has been published concerning the effects of other commonly used anticoagulants such as lithium heparin and potassium oxalate/sodium fluoride.



### **1.10 Use of Lp(a) measurements.**

At present, the use of serum Lp(a) measurements is limited. The analysis cannot be used as a screening test for coronary artery disease because assay protocols and calibration have not been standardised. Therefore, it may be that the assay should only be offered to patients attending specialist lipid clinics who have substantially raised plasma cholesterol concentrations, or evidence of premature coronary heart disease with no other identifiable risk factors. In the latter group, the finding of raised serum Lp(a) concentration might significantly increase risk status. It is also possible that certain apo(a) isoforms are more "atherogenic" than others <sup>(212)</sup>. Awareness of the significance of serum Lp(a) measurements is being increased through articles in international scientific journals such as Scientific American <sup>(213)</sup> which may stimulate the demand for quantitation of Lp(a). Eventually, if assay problems are overcome, the measurement of Lp(a) and its isoforms together with HDL and cholesterol, may become commonplace in the routine clinical chemistry laboratory.

### **1.11 Aims of the project.**

- 1 To select, produce and classify at least two anti-Lp(a) monoclonal antibodies.
- 2 To purify these antibodies and label them with europium.
- 3 To develop and optimise a time-resolved fluoroimmunoassay for Lp(a).
- 4 To assess the performance of the assay and compare it to the performance of other commercially available assays for Lp(a).
- 5 To assess possible interference in the assay by other biomolecules.
- 6 To use the assay to measure serum Lp(a) concentrations in various groups of patients.

## **CHAPTER 2**

### **MATERIALS AND METHODS**

## 2.1 REAGENTS AND MATERIALS.

### Microtitre plates.

Nunc-Immuno Module Maxisorp F16 Unframed Microtitre strips.

80 strips/box. 2 x 8 wells/strip. Catalogue number. 469914.

Nunc, Roskilde, DENMARK. (fax 45 42 350105)

Wells are certified to have absorbance readings within +/- 0.005 absorbance units from the mean value.

## SOLUTIONS

Reagents were purchased from the following companies.

### BAYER plc (Technicon)

Evans House,  
Hamilton Close,  
Basingstoke,  
Hampshire,  
U.K.  
RG21 2YE.

### MERCK Ltd (BDH).

Marketing supplies (BDH lab supplies),  
Merck House,  
Poole,  
Dorset, UK.  
BH15 1BR.

### SIGMA

The Sigma Chemical Co Ltd.  
Fancy Road,  
Poole,  
Dorset, UK.  
BH7 7NH.

### ICN BIOMEDICALS Ltd.

Lincoln Road,  
Cressex Industrial Estates,  
High Wycombe,  
Bucks, U.K.  
HP12 3XJ.

### Bicarbonate buffer, 0.1 M, pH 9.2

NaHCO <sub>3</sub>	8.40 g/L of deionised water.
Na <sub>2</sub> CO <sub>3</sub> (anhydrous)	10.60 g/L of deionised water.

Add 100 ml of Na<sub>2</sub>CO<sub>3</sub> solution to 900 ml NaHCO<sub>3</sub> solution. Adjust to pH 9.2 if necessary.

Store at room temperature.

**Phosphate-buffered saline (PBS) pH 7.4**

Solution is made up as a concentrate and diluted 25-fold for use.

Na <sub>2</sub> HPO <sub>4</sub> .12 H <sub>2</sub> O	73.4 g
Na H <sub>2</sub> PO <sub>4</sub> (anhydrous)	5.4 g
NaCl	219 g

Make up to 1.0 litre with deionised water and adjust pH to 7.4 if necessary.

Dilute this stock solution 1 in 25 in deionised water for use.

Store at room temperature.

**Citrate buffer, 0.1 M, pH 4.0.**

Trisodium citrate	29.41 g/L of deionised water.
Citric acid (anhydrous)	21.0 g/L of deionised water.

Add 410 ml of trisodium citrate solution to 590 ml of citric acid solution. Adjust pH to 4.0 if necessary.

**PLATE 'BLOCKING' REAGENTS**

**Bovine Serum Albumin**

Sigma - code A-7030

Fatty acid-free.

**Human Serum Albumin**

Sigma - code A-1887

Essentially fatty acid-free (approx 0.005%) Prepared from fraction V albumin.

**Bovine Gamma Globulin**

ICN code - 820412

**ANTIBODY SCREENING ASSAY REAGENTS**

**Supernatant diluent.**

PBS + 1% (w/v) bovine serum albumin (BSA).

Store frozen in 40 ml aliquots at -20°C.

**Wash Buffer.**

PBS + 0.5% (v/v) Tween 20 detergent.

Store at room temperature.

**Peroxidase-Antibody conjugate diluent.**

PBS + 1% (w/v) BSA + 0.1% (v/v) Tween 20.

Make up fresh daily.

**ABTS solution.**

ABTS = 2, 2' - Azino bis (3 - ethylbenzthiazolinesulfonic acid) Sigma code A-1888

Dissolve 1.0 g of ABTS in 1.0 L of 0.1 M Citrate buffer pH 4.0.

Divide into 50 ml portions. Stored at -20°C the reagent is stable for at least 1 year .

**Hydrogen peroxide solution.**

Sigma code H-1009

30% solution. Store at 4°C.

**Working Assay Substrate.**

Dilute the hydrogen peroxide 1:100 in deionised water.

Just before use add 10µl diluted H<sub>2</sub>O<sub>2</sub>/ml ABTS.

## OTHER CHEMICALS

### Bilirubin

Sigma - code B 4126

Crystalline preparation from bovine gall stones. Store dessicated below 4°C.

### Plasminogen

Sigma - code P-5661

## DETERGENTS

### Benzethonium chloride

Sigma - code B 8879

### Brij 99 (20 Oleyl ether)

Technicon - Supplied as a 20% (v/v) solution.

### Cholic acid (sodium salt)

Sigma - code C 1254

### CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1 propane sulfonate.

Sigma - code C 3023

### Deoxycholic acid (sodium salt)

Sigma - code D 6750

### Tween 20 (Polyoxyethylenesorbitan monolaurate)

BDH - code P 1379

### Triton X-100 (Polyoxyethylene ethers)

BDH - code 30632

### Triton X-115, X-405

Sigma

### Wetting Agent W

Technicon - code T21-1298

## CHROMATOGRAPHY REAGENTS

### Sephadex G-25

Sigma code G-25-150

### Sepharose 6B

Sigma code 6B-100

### Hydroxyapatite (calcium phosphate hydroxide - type I)

Sigma code H-0252

## TIME-RESOLVED FLUOROIMMUNOASSAY (DELFLIA®) REAGENTS

### DELFLIA Labelling buffer : 50 mmol/l NaHCO<sub>3</sub>, 0.9% (w/v) NaCl, pH 8.5.

NaHCO<sub>3</sub> 4.20 g

NaCl 9.0 g

Make up to 1.0 litres with deionised water. Adjust pH to 8.5 if necessary.

### DELFLIA Elution Buffer : 50 mmol/l Tris HCl, 0.9% (w/v) NaCl, pH 7.8.

Trizma Base 6.065 g

NaCl 9.0 g

Sodium Azide (NaN<sub>3</sub>) 0.5 g

Dissolve in 800 ml of deionised water, adjust pH to 7.8 with concentrated hydrochloric acid. Make up to 1.0 L with deionised water.

### DELFLIA Column decontamination buffer : 10 mmol/l potassium hydrogen phthalate, 0.001 % DTPA, pH 4.0.

Potassium hydrogen phthalate 2.04 g

DiethyleneTriamine-Pentaacetic Acid (DTPA) 0.01 g

Make up to 1.0 litres with deionised water, adjust pH to 4.0 if necessary.

These reagents are stable for at least 6 months at room temperature if bacterial contamination is avoided.

### DELFLIA Assay Buffer, Wash Buffer & Enhancement solution.

These solutions are supplied by Wallac in their commercial assay kits; additional reagents may be purchased individually from Wallac if required.

Certain component concentrations are not stated by the manufacturer.

#### DELFLIA Assay Buffer

Tris-HCl Buffer, pH 7.8 1.0 litres

Bovine serum albumin 5.0 g

Bovine globulin 0.5 g

Tween 40 0.2 ml

DTPA

Inert red dye. Store at 4 °C.

#### DELFLIA Wash buffer

Tris-HCl buffer, pH 7.8

Tween 20

Germall II (preservative)

Supplied as a 25-fold concentrate. Dilute with deionised water for use.

Store at 4 °C.

#### DELFLIA Enhancement solution

A buffer of pH 2-3 containing:-

2-naphthoyl-trifluoroacetate

Trioctylphosphine oxide

Triton X-100

Store at room temperature away from direct sunlight.

### WALLAC (U.K) Ltd.

Davy Avenue,

Knowlhill,

Milton Keynes

MK5 8PH.

## COMMERCIAL ANTIBODIES & STANDARD

### THE BINDING SITE LIMITED.

Institute of Research and Development,  
Birmingham Research Park,  
Vincent Drive,  
Birmingham, UK.  
B15 2SQ.

#### Anti-Human Apolipoprotein B.

The Binding site - Economy grade; code PE086 (lot A6718)  
Cyto grade (IgG fraction) ; code PC086 (lot A4419)

Antibody developed in sheep, economy grade contains whole antiserum; cyto grade has undergone solid phase adsorption and has a total protein content of 10-20 mg/ml.

#### Anti-Human Apolipoprotein B peroxidase conjugate.

The Binding Site - code PP086 (batch G2669)

Cyto grade antiserum conjugated with horseradish peroxidase in Tris/saline buffer, pH 7.4, immunoglobulin : enzyme molar ratio of 1:1.

### IMMUNO LTD.

Arctic House,  
Rye Lane,  
Duncton Green,  
Sevenoaks,  
Kent, UK.  
TN14 5HB

#### Anti-Human Lp(a) antiserum.

Immuno - code 4845005  
Antibody developed in sheep.

#### Human Lp(a) reference standard.

Immuno - code 4395005

Lyophilised, defibrinated pooled human plasma with stabilisers added to prevent irreversible destruction of apo B-containing proteins during the freeze drying process. Concentration assigned by comparison with a primary standard of purified Lp(a).

The standard was reconstituted with 0.5 ml of deionised water using a Gilson pipette checked for accuracy and precision. After mixing on a roller-mixer for 30 minutes the standard was divided into 30µl portions and stored frozen at -20°C.

The frozen standards were stable for at least three months.

### DAKO Ltd.

16 Manor Courtyard,  
Hughenden Avenue,  
High Wycombe,  
Bucks, U.K.  
HP13 5RE

#### Donkey Anti-sheep IgG peroxidase conjugate.

Dako - code P163 (lot number 39)

#### Sheep Anti-mouse IgG (whole molecule) peroxidase conjugate (affinity purified).

Sigma immunochemicals - code A-5906 (lot number 040H8800)

## 2.2 COMMERCIAL Lp(a) ASSAY KITS.

The following kits were commercially available in the United Kingdom at the time of this study. Each kit contained sufficient reagents to assay approximately 35 patient samples in duplicate. The cost of each kit was ~£200.

### ELISA ASSAYS.

#### *Immuno Immunozyt® kit.*

#### IMMUNO LTD.

Arctic House,  
Rye Lane,  
Duncton Green,  
Sevenoaks,  
Kent, UK.  
TN14 5HB

This kit <sup>(214)</sup> uses a one-step two-site assay protocol. Microtitre plates coated with a polyclonal anti-apolipoprotein (a) antibody are used to capture Lp(a) which is then detected by a horseradish peroxidase-conjugated monoclonal anti-apolipoprotein (a) antibody. Although Immuno state that the antibodies recognise the kringle 4 domain of Lp(a) they claim that this does not affect quantitative measurements <sup>(215)</sup>.

The assay uses a 1:500 dilution of sample which is added to the antibody-coated microtitre plate together with the peroxidase-conjugated antibody. After a 1 hour incubation at 20°C, the microtitre plate is washed four times and a substrate, tetramethylbenzidine (TMB), is added. The substrate is incubated for 30 minutes at room temperature. TMB is then cleaved by horseradish peroxidase to produce a blue coloured product which turns yellow on addition of sulphuric acid which also stops the reaction. The absorbance of the solution at 450 nm is then measured and used to generate a standard curve from which the unknowns may be calculated.



***Innogenetics Innotest Lp(a) kit.***

**INNOGENETICS.**

Kronenburgstraat 45,  
B 2000  
Antwerp,  
Belgium.

This kit <sup>(216)</sup> uses a monoclonal anti-apolipoprotein (a) capture antibody and a horseradish peroxidase-conjugated anti-apolipoprotein B detection antibody. The assay is performed in two steps. In the first step, samples diluted 1:2000 in assay buffer are incubated with the capture antibody for two hours at 37°C. The microtitre plate is then washed and detection antibody is added for 1 hour at 37°C. The assay is then completed using a procedure identical to that of the Immunozy<sup>®</sup> assay.

***Biopool TintElize Lp(a) kit.***

**PORTON CAMBRIDGE Ltd.**

Lanwades Business Park,  
Kennett,  
Newmarket, Suffolk, U.K.  
CB8 7PW

This kit <sup>(217)</sup> uses anti-apolipoprotein (a) antibodies (type not specified) as both capture and detection antibodies. Samples are diluted 1:2601 and incubated with capture antibody for 2 hours at room temperature. Without washing the plate, 50µl of horseradish peroxidase-conjugated antibody is added to each well and incubated for a further 1 hour at 25°C. The plate is then washed and a coloured product is generated as in the other Lp(a) ELISA assays. This assay kit is unusual in that the Lp(a) standards are supplied in lyophilised form incorporated into the well of the microtitre plate.

## IMMUNORADIOMETRIC ASSAY

### *Pharmacia apo(a) IRMA.*

This is a two-site immunoradiometric assay <sup>(218)</sup> using two anti-apolipoprotein (a) monoclonal antibodies directed against different antigenic determinants of apolipoprotein (a). One of these antibodies is labelled with <sup>125</sup>I and the other is bound to a solid phase (sepharose particles). Before analysis, samples are mixed at a 1:1 ratio with a pretreatment solution (thought to contain a reducing agent) which releases apolipoprotein (a) from the Lp(a) particle. After the incubation step, the sample is further diluted 1:21 in a buffer. The diluted sample is then incubated simultaneously with both antibodies for 1 hour at 20°C. Immune complexes are precipitated with polyethylene glycol and centrifuged to produce a pellet. The liquid supernatant is then decanted and the gamma radiation in the pellet is measured.

Unlike the ELISA assays, the IRMA assay is calibrated in terms of apolipoprotein (a) units/L and the manufacturers state that, although no direct conversion can be made to mass units due to variations in the size of apolipoprotein (a) isoforms, an approximate conversion factor of 1 unit of apo (a) = 0.7 mg Lp(a) may be applied.

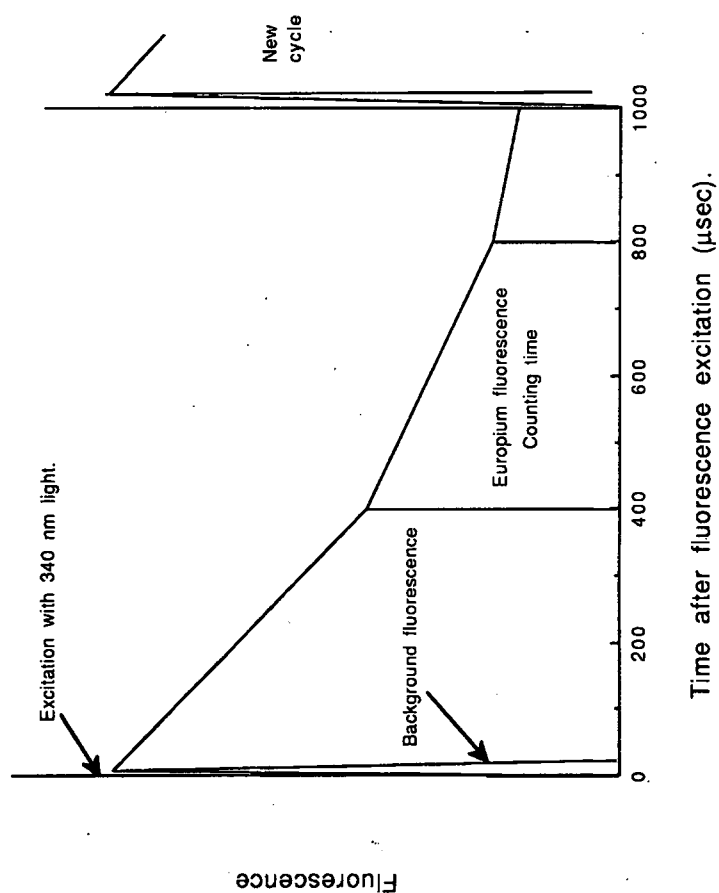
## **2.3 ABSORBANCE MEASUREMENTS in MICROTITRE PLATES.**

Absorbance readings were made using the V-max microtitre plate reader (Novo Biolabs) linked to an Acorn Archimedes computer running Novoclone® microtitre plate reading software. Monochromatic light was produced by means of filters, the selection of which is controlled by the computer software. To make an absorbance reading, the microtitre plate is simply inserted into the instrument and readings commenced under software control. The absorbances of the 96 wells of a microtitre plate may be measured in less than 30 seconds. End-point readings were taken for all assays in this project. The absorbance data obtained may be printed out in its raw form or used to generate a standard curve and calculate unknowns.

## **2.4 MEASUREMENT OF TIME-RESOLVED FLUORESCENCE (219).**

Conventional fluorescence assays have severe limitations in sensitivity due to high background fluorescence from biomolecules (especially proteins) and light scattering effects. The time-resolved fluorescence technique uses a label whose fluorescence is measured after a delay time during which background fluorescence has decayed completely. This label is europium, a member of the lanthanide series of elements, which has a very long fluorescence decay time and a large Stokes' shift (i.e. the difference between the excitation wavelength of 340 nm, and the emission wavelength of 608 nm). In addition, the emission peak is of a very narrow band of wavelengths.

Most fluorescent compounds (fluorophores) have decay times of less than 10 nsec. In contrast, europium has a decay time of 730  $\mu$ sec. Therefore, if fluorescence readings are taken at a time interval of between 400-800  $\mu$ sec, no background fluorescence will be measured. In the time-resolved fluorimeter, europium is pulsed with light at the excitation wavelength for 1000 cycles/second. In the period between these pulses, fluorescence is measured and the counts from each reading are accumulated. A diagrammatic representation of this procedure is presented in figure 2.1.



**Figure 2.1** Time-resolved fluorescence :  
Schematic diagram of fluorescence production during one measurement cycle.

A typical time-resolved fluorescence assay may generate in excess of 1 million fluorescence counts for standards at the highest analyte concentration and less than 1000 counts for the zero standard. This enables highly sensitive assays to be produced with a working range up to 5 orders of magnitude.

Fluorescence readings are made using the DELFIA® 1234 research fluorimeter. The practical aspects of the procedure are very simple. Following release of europium from the secondary antibody by addition of enhancement solution, the microtitre plate is loaded into the measurement chamber of the instrument, exposed to monochromatic light at the fluorescence excitation wavelength of 340 nm (1000 pulses/sec), and the emitted fluorescence is detected. The fluorescence counts data may be sent to a data handling software package such as Pharmacia's Multicalc or taken directly from the fluorimeter. The instrument is pre-set to read fluorescence emission from each well for 1 second. This time cannot be adjusted and, after reading one well, movement onto the next well is made automatically. 12 x 8 microtitre plates are read in a specific way, rows of 12 wells are always read in a left-to-right direction which cannot be adjusted. Consequently assays should be set up with standards along the top 12 wells of the microtitre plate so that they are read first. The time taken to read a complete 96 well plate is around 3 minutes. If required, the microtitre plate may be read several times since fluorescence (according to the manufacturers) is stable for up to 24 hours if evaporation of solutions is avoided.

## **2.5 ASSAYS ON THE DuPONT DIMENSION®AR ANALYSER**

The following analytes were measured on the DuPont Dimension® AR autoanalyser, all reagents were supplied in the flex® reagent pack used on the instrument. The instrument performance was checked by analysis of quality control materials.

### **Total Bilirubin.**

The method is a modification of the Jendrassik and Grof procedure <sup>(220)</sup>. Bilirubin in solution is solubilised by dilution in a solution of caffeine/sodium benzoate/acetate and disodium EDTA. Diazotised sulfanilic acid (formed by combining sodium nitrite and sulfanilic acid at low pH) is added to the bilirubin which is then converted to diazo-bilirubin, a red chromophore, which absorbs light at a wavelength of 540nm. Bichromatic 'blanking' is performed by taking a second absorbance measurement at 700nm. The absorbance increase is proportional to bilirubin concentration in the sample, all forms of bilirubin including the conjugated, unconjugated and delta forms are measured.

### **Total Protein.**

The method is based on Henry's modification <sup>(221)</sup> of the biuret reaction first introduced by Kingsley <sup>(222)</sup>.

Cupric ion ( $\text{Cu}^{++}$ ) reacts with peptide bonds of proteins at an alkaline pH and a blue copper (II) protein complex is formed, the colour intensity of which is proportional to the total protein concentration of the sample. Absorbance of solutions are read at 540nm and at a bichromatic blanking wavelength of 700nm. Absorbance is proportional to protein concentration in the sample under test. Sodium potassium tartrate is added to the reagent to prevent the formation of insoluble copper (II) hydroxide.

### **Albumin**

Albumin was measured by a dye-binding technique described by Carter and Louderback *et al* <sup>(223)</sup>. The method is more specific for human albumin than other dye binding procedures. In acetate buffer pH 4.9 containing a surfactant solubilising agent, albumin binds to the dye Bromocresol Purple (BCP); the albumin-BCP complex absorbs light at 600nm. The absorbance is proportional to the albumin concentration of the sample under test.

**CHAPTER 3**  
**GENERATION and PURIFICATION**  
**OF ANTI-Lp(a) MONOCLONAL ANTIBODIES**

The monoclonal antibodies used in this project were generated by Drs. T. Wooley and T. Wang (Dept. Clinical Biochemistry, Cambridge.) by immunisation of mice with a highly purified preparation of Lp(a) (Immuno). A panel of antibody-secreting cell lines thus generated were characterised using a series of immunoassays designed by the author in conjunction with Dr. Wang. The production and collection of mouse ascitic fluid was performed by Dr. Wooley.

Two independent antibody screening assays were initially employed to determine (a) whether the cell culture supernatants contained any antibodies, and (b) whether these antibodies were directed against Lp(a). In subsequent screening assays the antibodies were used in competition with other antibodies of known specificity in an attempt to determine whether they were directed against epitopes on the apo B or apo (a) regions of Lp(a).

### **3.1. SCREENING FOR THE PRESENCE OF ANTIBODIES IN CELL CULTURE SUPERNATANTS.**

#### **3.1.1 Method 1 (figure 3.1)**

Microtitre plates were coated with a 1:3400 dilution of an 800 mg/L Lp(a) standard preparation (Immuno) which contains both Lp(a) and other human plasma proteins in 0.1M bicarbonate buffer, pH 9.2. 150µl of this solution was used to coat each well during an overnight incubation at 4°C. During this incubation both Lp(a) and other proteins bound non-specifically to the plastic of the well (a process known as 'passive diffusion' (224)). After coating, the plate was washed four times with a wash solution of phosphate-buffered saline (PBS) containing 0.5% (v/v) Tween 20 detergent to remove unbound standard.

Cell culture supernatants were diluted 1:10, 1:100 and 1:1000 in supernatant diluent (which consisted of PBS supplemented with bovine serum albumin (BSA) at a concentration of 10 g/L). 100µl aliquots of diluted supernatant were pipetted, in duplicate, into the Lp(a)-coated microtitre plate wells. Sheep anti-human Lp(a) antiserum (Immuno) was used as a positive control, while fresh cell culture medium



(containing no antibodies) was used as a negative control. The microtitre plate was covered and incubated overnight at 4°C.

The microtitre plate was then washed four times and 125µl of a 1:250 dilution (in supernatant diluent/0.1% (v/v) Tween 20) of an ovine anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Sigma Immunochemicals) was pipetted into wells. As a positive control, 125µl of donkey anti-sheep IgG-HRP conjugate (1:1000) was added to replicate wells. The microtitre plate was incubated at room temperature for 1 hour, washed four times, and 150µl of the HRP substrate, ABTS (see Materials and Methods) was pipetted into each well. The microtitre plate was incubated at room temperature in the dark until the absorbance of the most intensely coloured well (measured at 490 nm using a V-max microtitre plate reader) was greater than 1.0, but less than 2.0. A 'blank' absorbance reading (taken at 405 nm) was subtracted from the reading at 490 nm.

This screening method was designed to determine whether the cell culture supernatants contained any anti-human antibodies (of any specificity). Since the microtitre plate was coated with other human plasma proteins (in addition to Lp(a)), any mouse antibodies present in the cell culture supernatants that subsequently bound to the wells could be directed against either human Lp(a) or any of the other human plasma proteins present.

## Results.

In excess of 100 cell culture supernatants were screened using the procedure outlined above. Those supernatants giving the greatest absorbances are shown below. An absorbance greater than 0.2 indicates the presence of a mouse anti-human antibody.

<u>Cell culture supernatant.</u>	<u>Absorbance (490-405 nm)</u>
1/7/A9	1.142
2/9/C10	1.053
3/2/B5	1.021
1/7/E4	0.995
3/5/G5	0.965
4/1/H12	0.944
2/10/F4	0.924
1/8/F5	0.918
3/2/A11	0.770
1/7/G12	0.722
Negative control	0.100

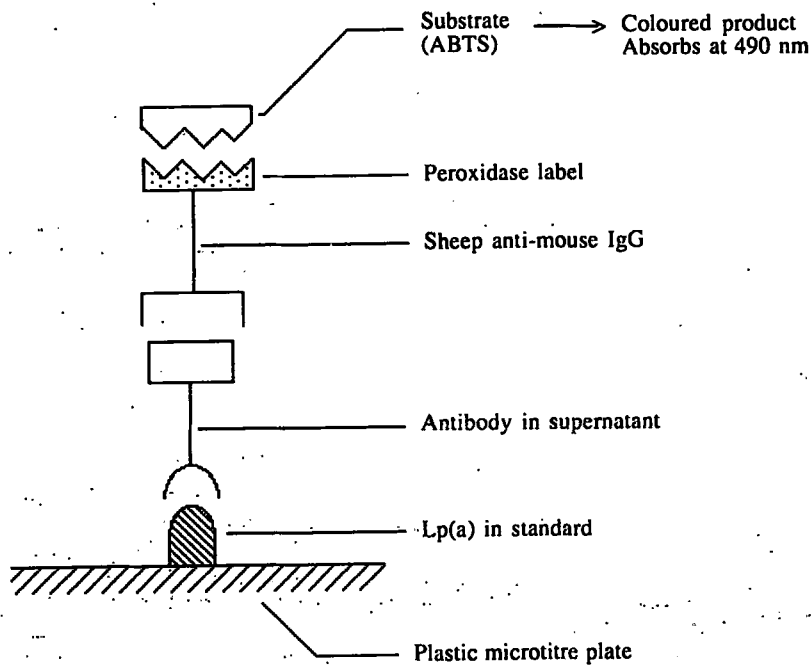
Cell culture supernatants with an absorbance below 0.2 were eliminated from the next series of screening tests.

### 3.1.2 Method 2 (figure 3.2)

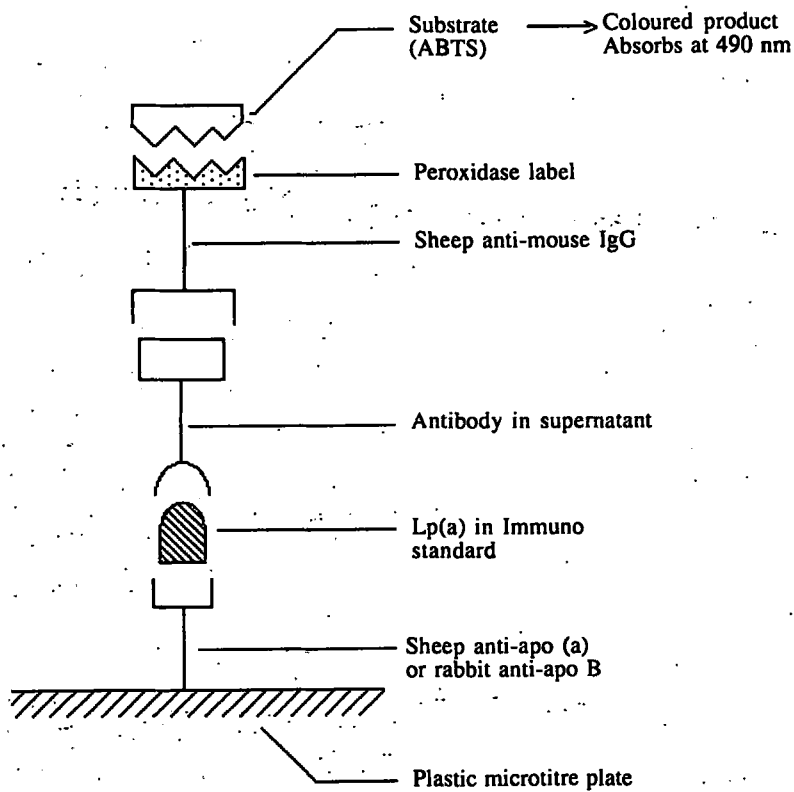
A two-site 'sandwich' assay using commercially-available antibodies directed against Lp(a) (either sheep anti-human apo B from 'The Binding Site' or sheep anti-human apo (a) from Immuno) was used to screen for the presence of anti-Lp(a) antibodies. 150µl of either the anti-apo B antibody (diluted 1:100) or the anti-apo (a) antibody (diluted 1:1000) were used to coat the microtitre plate by passive diffusion overnight at 4°C. Dilutions were made in bicarbonate buffer, pH 9.2. After incubation, the plate was washed four times and 75µl of a 1:3400 dilution of Lp(a) standard (850 mg/L) in supernatant diluent was added to each well. The microtitre plate was covered and incubated at 4°C overnight.

The following day the plate was washed four times and cell culture supernatants (diluted 1:10, 1:100 and 1:1000 in supernatant diluent), positive and

**FIGURE 3.1 :**  
**Antibody screening method 1**



**FIGURE 3.2 :**  
**Antibody screening method 2**



negative controls were added to the wells. Subsequently the assay employed the same protocol as method 1.

High absorbance indicate the presence in cell culture supernatants of antibodies directed against of Lp(a), although the method is unable to differentiate anti-apo (a) and anti-apo B specificities.

## **Results.**

A series of ~100 cell culture supernatants (which were positive in method 1) were screened. The absorbances obtained for each supernatant using either anti-apo (a) or anti-apo B coated microtitre plates are presented below.

### **Anti-apo (a) antibody.**

<u>Cell culture supernatant</u>	<u>Absorbance (490-405 nm)</u>
3/5/F5	1.196
2/9/C10	1.135
1/H5	1.118
1/H12	1.058
4/1/H12	1.058
1/8/F5	0.936
2/10/F4	0.635
1/7/G12	0.233
1/7/E4	0.201
3/2/F5	0.134
1/7/A9	0.116
3/2/A11	0.091
Negative control	0.090

### **Anti-apo B antibody.**

<u>Cell culture supernatant</u>	<u>Absorbance (490-405 nm)</u>
3/5/F5	0.365
2/9/C10	0.349
1/H12	0.332
1/H5	0.302
1/8/F5	0.292
2/10/F4	0.287
4/1/H12	0.274
1/7/A9	0.124
1/7/G12	0.091
Negative control	0.090

Absorbances for the other supernatants were less than 0.1.

The use of these two screening tests (methods 1 & 2) allowed the rapid elimination of a large proportion of the panel of several hundreds of cell culture supernatants that might provide antibodies for Lp(a) immunoassays. It may be noted that some of the supernatants that were strongly positive in method 1 (1/7/A9 and 3/2/B5) were only weakly positive in method 2, suggesting these were not anti-Lp(a) antibodies. However, two supernatants, 3/5/F5 and 2/9/C10 (35f5/29c10) consistently produced high absorbance readings in each method. Aliquots of each of these cells were therefore injected into the peritoneal cavities of mice in order to generate monoclonal antibody-containing ascitic fluid. These ascitic fluids (containing either 29c10 or 35f5 anti-Lp(a) antibodies) were used in the next series of screening tests.

## **3.2 DETERMINATION OF THE SPECIFICITIES OF ANTI-Lp(a) ANTIBODIES.**

A series of assays were developed with an aim of identifying the epitopes of Lp(a) recognised by the monoclonal antibodies. The assays employed competition between the monoclonal antibodies and commercial antibodies of known specificities for binding sites on Lp(a).

### **3.2.1 Method 3 (figure 3.3)**

This two-site assay employs competition between a commercial polyclonal antibody (bound to the microtitre plate) and the test monoclonal antibody (in solution) for binding to added Lp(a) (also in solution). If the test antibody bound an epitope of Lp(a) recognised by the immobilised antibody then less Lp(a) would bind to the microtitre plate. The bound Lp(a) is subsequently detected using an HRP-conjugated antibody directed against a different Lp(a) epitope to that recognised by the immobilised antibody. Hence, competition between the test antibody and the immobilised antibody for the same Lp(a) epitope results in decreased absorbance. In principle, however, it may be possible for both the test and immobilised antibodies to bind to the same region of Lp(a) with no competition. This is because the immobilised polyclonal antibody has, by definition, more than one binding site on Lp(a).

Commercial polyclonal anti-apo (a) or anti-apo B antibodies were used to coat microtitre plates (as in screening method 2). After coating with antibody, 75µl of Lp(a) standard (250 ng/ml) and 50µl of ascitic fluid (diluted 1:100 - 1:10000 as in method 2) were pipetted into the wells and incubated overnight at 4°C. The assay was then completed as in screening method 2.

### **Results.**

The absorbances obtained at each ascitic fluid dilution for each antibody were the same (data not shown). This finding suggests that binding of the monoclonal antibody to Lp(a) did not inhibit its binding to either of the commercial polyclonal

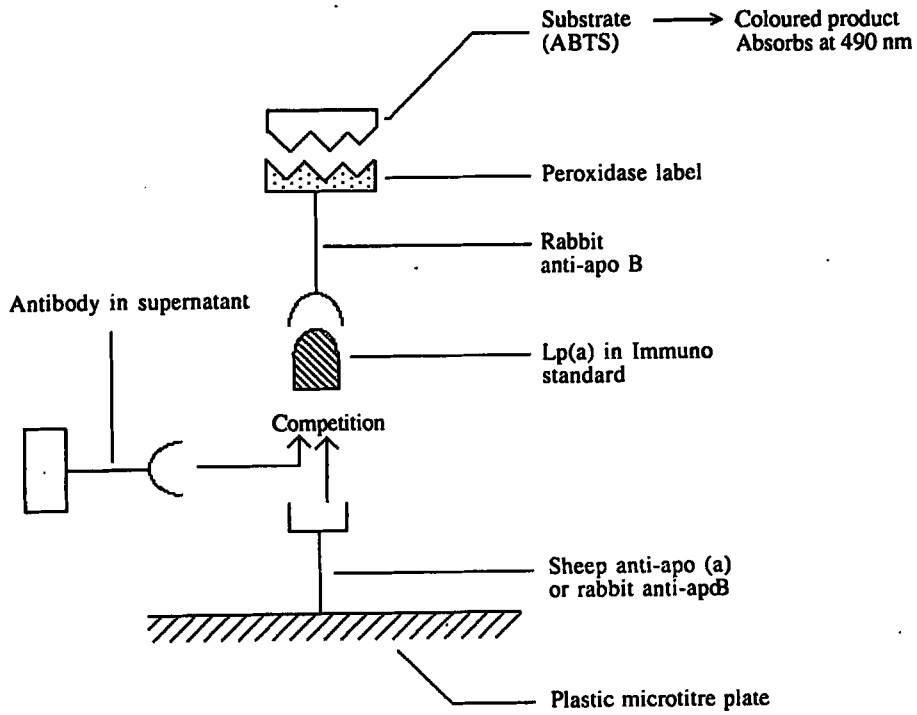
capture or detection antibodies. Variations in the reaction conditions of these assays also failed to demonstrate any competition between the immobilised antibodies and the test antibodies.

### **3.2.2 Method 4 (figure 3.4)**

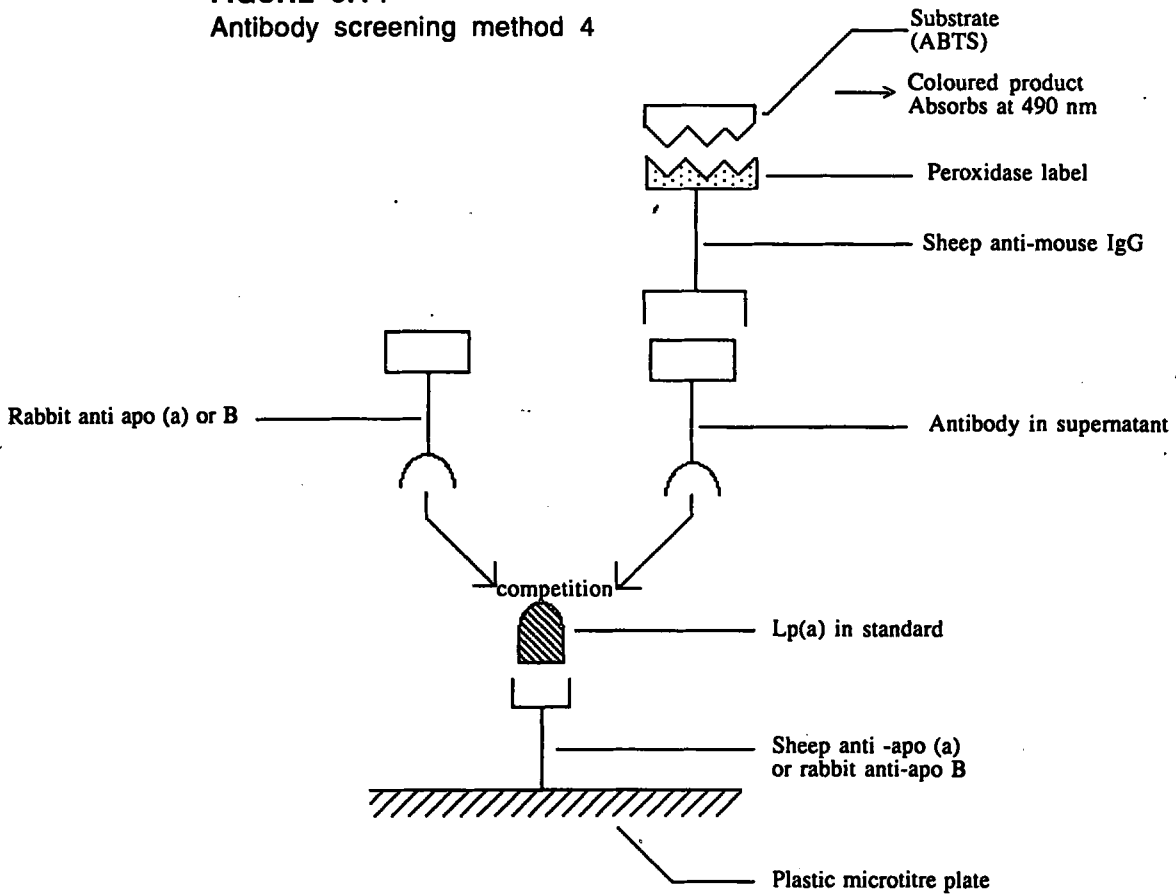
This assay employs microtitre plates coated with an anti-apo (a) polyclonal antibody to capture Lp(a) present in 75 $\mu$ l of Lp(a) standard (25 ng/ml) during an overnight incubation at 4°C. After washing, 50 $\mu$ l of mouse ascitic fluid (diluted 1:10000) was added to each well together with 50 $\mu$ l of the commercial polyclonal anti-apo (a) or anti-apo B antibodies (diluted 1:10, 1:100 or 1:1000). These antibodies were added to compete with the monoclonal antibody for binding sites on the immobilised Lp(a). In some wells the commercial antibody was replaced by 50 $\mu$ l of diluent only (blank), in order to provide an estimate of the maximum binding of the monoclonal antibody to the immobilised Lp(a). Following incubation of the reaction mixture at room temperature for 4 hours, the assay was completed using the standard protocol (section 3.1). The absorbance reading in each well was proportional to the amount of monoclonal antibody bound to the immobilised Lp(a).

The assay relies on competition between the polyclonal and monoclonal antibodies for binding sites on Lp(a). Hence, displacement of the polyclonal antibody (of known specificity) suggests that the test monoclonal antibody is directed against the same epitope.

**FIGURE 3.3 :**  
Antibody screening method 3



**FIGURE 3.4 :**  
Antibody screening method 4





## **Results.**

### **(i) 35f5 monoclonal antibody (ascitic fluid).**

#### **Absorbances in the presence of a polyclonal anti-apo (a) antibody.**

<b>Polyclonal antibody dilution</b>	<b>Absorbance (490-405 nm)</b>
1:10	0.084
1:100	0.132
1:1000	0.184
Blank (maximum binding of 35f5)	0.183

#### **Absorbances in the presence of a polyclonal anti-apo B antibody.**

1:10	0.172
1:100	0.185
1:1000	0.190
Blank (maximum binding of 35f5)	0.192

### **(ii) 29c10 monoclonal antibody (ascitic fluid).**

#### **Absorbances in the presence of a polyclonal anti-apo (a) antibody.**

<b>Polyclonal antibody dilution</b>	<b>Absorbance (490-405 nm)</b>
1:10	0.099
1:100	0.138
1:1000	0.180
Blank (maximum binding of 29c10)	0.187

#### **Absorbances in the presence of a polyclonal anti-apo B antibody.**

1:10	0.177
1:100	0.185
1:1000	0.180
Blank (maximum binding of 29c10)	0.196

The decreases in absorbance obtained when an excess (~50 µg) of anti-apo (a) polyclonal antibody was added to the reaction mixture suggests that both of the monoclonal antibodies are directed against epitopes on the apo (a) region of Lp(a). In principle, the addition of relatively large amounts of polyclonal antibody may affect the binding of the monoclonal antibody to Lp(a) in a non-competitive manner. However, this possibility seems unlikely since the anti-apo B polyclonal antibody (also at ~50µg) caused little or no displacement of the monoclonal antibody.

### **3.3 FURTHER CHARACTERISATION OF THE BINDING SPECIFICITIES OF 35f5 and 29c10.**

#### **3.3.1 Introduction.**

Possibly the simplest method of determining whether the monoclonal antibodies recognise epitopes on the anti-apo (a) or B regions of Lp(a) is to reanalyse serum samples found to have a high concentration of apo B and a low concentration of Lp(a) in assays using commercial anti-Lp(a) antibodies of known specificity. If, on reassay (after replacing one of these commercial antibodies with a monoclonal antibody), the samples give a completely different result, it is likely that the monoclonal antibody is directed against a different region of Lp(a) to that recognised by the polyclonal antibody it has replaced.

#### **3.3.2 Method.**

Analyses were performed using the time-resolved fluorescence assay (see chapters 5-7). Assays were developed using the following antibody combinations.

<b>Capture antibody</b>	<b>Detection antibody</b>
'Immuno' anti-apo (a)	'Binding Site' anti-apo B
'Binding Site' anti-apo B	'Binding Site' anti-apo B
35f5	'Binding Site' anti-apo B
29c10	'Binding Site' anti-apo B

The apo B standard (1005 mg/L) from the Pharmacia apo B IRMA kit was used in the assay. Analysis in the (a)/B time-resolved fluorescence Lp(a) assay showed it to contain a low concentration (~150 mg/L) of Lp(a). The standard was diluted 1:500 in assay buffer and subjected to doubling Dilutions to produce a series of standards. These standards, together with 7 serum samples, were analysed using each of the antibody combinations.

The fluorescence counts for each apo B standard were used to construct a standard curve for each of the antibody combinations. The apo B concentrations of the unknowns were estimated from these curves.

Results are presented in table 3.1

**Table 3.1 : Apo B concentrations (mg/L) for seven serum samples measured in time-resolved fluorescence assays using an anti-apo B detection antibody with each of four different capture antibodies.**

Sample	Capture antibody.			
	anti-apo (a)	anti-apo B	35f5	29c10
1	850	235	884	>1005
2	>1005	278	>1005	>1005
3	>1005	351	>1005	>1005
4	>1005	382	>1005	>1005
5	960	220	>1005	>1005
6	>1005	278	>1005	>1005
7	>1005	1036	>1005	>1005

It is apparent from these results that there is considerable disparity between the values obtained using the dual anti-apo B assay format (B/B) and those obtained using anti-apo (a)/anti-apo B antibody combination ((a)/B). Results using the monoclonal antibodies were similar those generated using the (a)-B assay format.

These discrepancies may be explained as follows: the B-B assay measures apo B in both standards and unknowns and apo B present in Lp(a). In the (a)-B assay, the presence 1005 mg/L of apo B in the standard will not produce a fluorescence signal as apo B cannot bind to the capture antibody. However, the 150 mg/L of Lp(a) present in the undiluted standard will bind to the capture antibody, as will any Lp(a) present in the serum samples. If these serum samples had Lp(a) concentrations in excess of 150 mg/L, the fluorescence signals would be greater than that of the standard, hence the 'apparent' apo B concentration would be greater than the actual apo B concentration in the standard (1005 mg/L). It may be seen that this occurred for 6 out of 7 of the serum samples. The assays using 29c10 or 35f5 capture antibodies showed a similar result pattern to the (a)-B assay. This was taken to be further evidence that the antibody was directed against an epitope on the apo (a) region of Lp(a). Further studies on the characterisation of the monoclonal antibodies will be presented in chapter 5.

### **3.4. ANTIBODY PURIFICATION.**

#### **3.4.1 Introduction.**

Approximately 5 ml of mouse ascitic fluid containing either the 29c10 or 35f5 monoclonal antibody were collected. Since these fluids are likely to contain other proteins (particularly albumin) which would also be labelled during the europium labelling procedure (chapter 4), initial purification of the antibody was necessary.

#### **3.4.2 Methods.**

##### **Ammonium sulphate precipitation.**

3.15 ml of saturated ammonium sulphate solution was added to 4.5 ml of ascitic fluid (containing either 29c10 or 35f5) and the mixture was stirred continuously for 30 minutes at room temperature. Following centrifugation at 10,000 g for 20 minutes at 20°C, the supernatant was discarded and the precipitated protein pellet was resuspended in 1.0 ml of 80 mmol/L phosphate buffer. The suspension was

then dialysed overnight at 4°C against 80 mmol/l phosphate buffer to remove the ammonium sulphate.

### **Hydroxyapatite chromatography.**

The dialysate containing 29c10 antibody was further purified by adsorption chromatography using a hydroxyapatite column eluted with a phosphate buffer gradient (10-250 mmol/L). Eighty-four fractions of 2 ml were collected using an automated fraction collector (Pharmacia), and the absorbance of the eluate was continuously monitored at 280 nm. A plot of absorbance at 280 nm versus fraction number is shown in figure 3.5. The eluants were used to coat microtitre plates for Lp(a) assay (see chapter 4). These assays confirmed that fractions in the first peak eluted from the column were free of anti-Lp(a) antibodies while the larger second peak contained the 29c10 antibody. Thirty fractions (2 ml each) corresponding to the antibody peak were pooled and the absorbance of this solution was measured at 280 nm. The approximate protein concentration of the pool was estimated using the equation <sup>(182)</sup>:

$$\text{IgG (mg/ml)} = \frac{\text{Abs 280 nm}}{1.34}$$

The pooled antibody solution was concentrated using an Amicon concentration system employing a membrane which is permeable to molecules of less than 30 kD, but is impermeable to larger molecules. Using this technique, the 60 ml of pooled antibody solution was concentrated to a final volume of ~2 ml and the protein content was estimated by absorbance measurement at 280 nm. Surplus antibody not required for the europium labelling procedure was stored in 0.5 ml aliquots at -20°C after addition of sodium azide to 0.1% (w/v) as preservative.

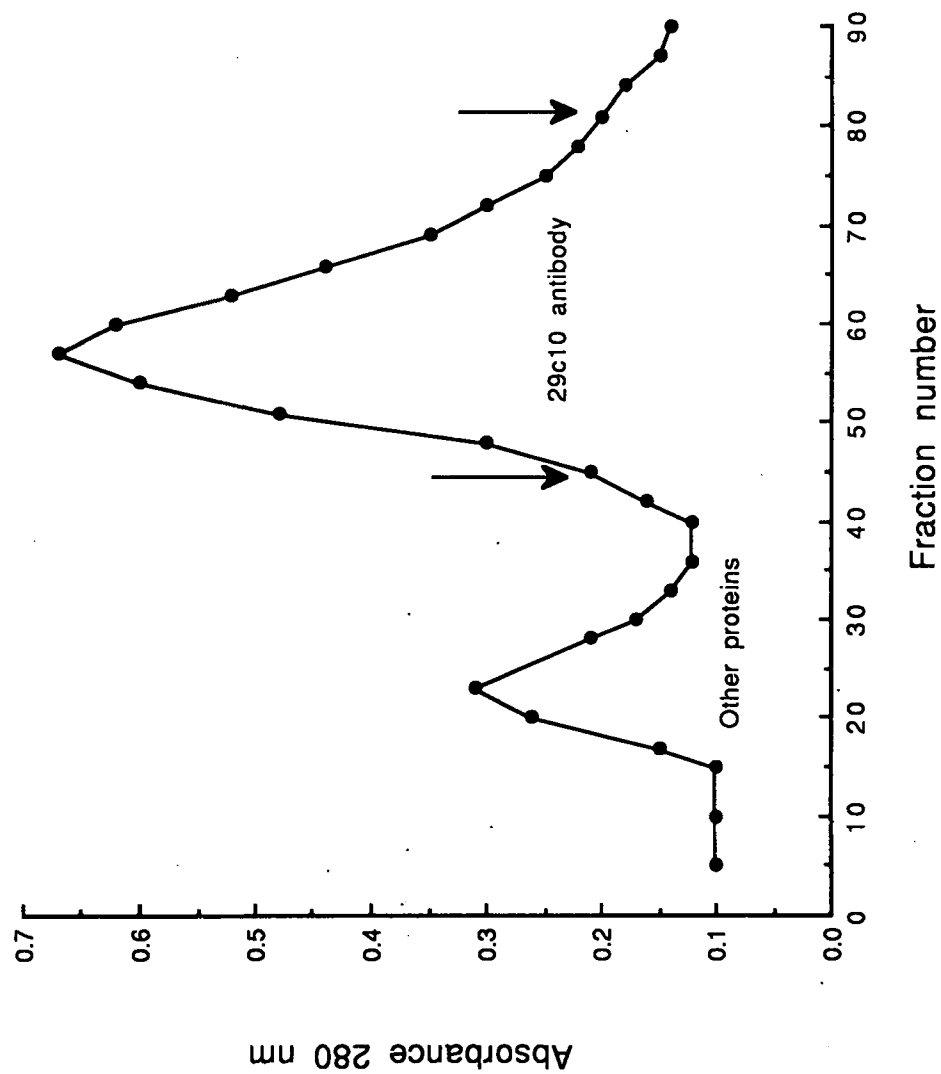
### **3.4.2 Results.**

Addition of ammonium sulphate to ascitic fluid should precipitate large proteins (e.g. antibodies) but not smaller proteins (e.g. albumin) which should remain in solution. However, the two absorbance peaks of the eluants from the

hydroxyapatite column (figure 3.5) suggests that some smaller proteins had also been precipitated. The 60 ml of pooled 29c10 antibody contained 13 mg of protein at 0.22 mg/ml. The protein concentration was increased by filter concentration to 7.2 mg/ml. Since the total volume of the concentrated antibody solution was 1.7 ml, it is apparent that 94% of the protein present in the original fractions was recovered.

### **3.5 CONCLUSIONS.**

A series of mouse hybridoma culture supernatants were screened using enzyme-immunoassays to identify cell lines producing anti-Lp(a) antibodies. However, these screening assays were unable to determine the epitope of Lp(a) recognised by the antibodies. The latter was achieved by comparison of fluorescence counts obtained for a serum sample (with a high apo B concentration and a low concentration of Lp(a)) measured using the time-resolved fluorescence assay with a series of antibodies of known specificities. Two antibodies (29c10 and 35f5) both directed against apo (a) were purified for use in Lp(a) immunoassays.



**Figure 3.5** Elution profile from hydroxyapatite adsorption chromatography of ascitic fluid containing monoclonal antibody 29c10. Fractions between the arrows were pooled and concentrated.

**CHAPTER 4**  
**PRACTICAL ASPECTS OF LANTHANIDE LABELLING**



## 4.1 Introduction.

All immunoassays employ a label that may be detected, quantitatively or qualitatively, by physical or chemical means. In a competitive immunoassay the (tracer) antigen is labelled, whereas in a two-site (or "sandwich") immunoassay an antibody is labelled. Conventional labelling materials include radioisotopes such as  $^3\text{H}$  or  $^{125}\text{I}$  (emitting beta and gamma radiation respectively) which may be detected by means of scintillation counters. Several radiolabelling procedures have been developed (225). These techniques are easy to perform but require expertise to consistently produce labels with the desirable specific activity. Many commonly-used radiolabelled antigens and antibodies are commercially available, although for research purposes, it is inevitable that some proteins (particularly those with little commercial demand) will need to be labelled in-house. Strict regulations control the use and disposal of radioisotopes in the United Kingdom. These state that iodinations (the incorporation of  $^{125}\text{I}$  into proteins), during which high intensity radiation is used, should be performed only in a designated room with lead shielding to protect the operator. Equipment is required to monitor the radioactive emissions and strict adherence to analytical protocols should be enforced to ensure that spillages and contamination of work areas is minimised. Any waste materials from the process also require special disposal procedures. Persons performing iodinations should undergo regular thyroid scans to ensure that  $^{125}\text{I}$  has not been absorbed and concentrated in the gland.  $^{125}\text{I}$  is the most commonly-used radioisotopic label. Proteins labelled with this isotope have a limited shelf-life (~1-2 months) due to radiation damage of the protein itself and loss of radioactive emission intensity due to the short half life (6 weeks) of  $^{125}\text{I}$ .

Radioisotope stability and safety problems have encouraged the use of alternative non-isotopic materials as labels in immunoassays such as enzymes, fluorophores (molecules which absorb light at one wavelength and re-emit light at a higher wavelength) and chemiluminescent compounds (molecules which emit light during an oxidative chemical reaction). Enzymes are widely employed: for example,

alkaline phosphatase and horseradish peroxidase are frequently used in commercial ELISA kit assays. It is possible to label proteins with enzymes 'in-house' but the procedures are more complex than iodinations. In the authors experience of in-house enzyme labelling, there has been a tendency for the enzyme to dissociate from its protein during prolonged (2-3 months) storage at 4°C which increases non-specific binding and assay imprecision. The enzyme acts on an added substrate to generate a coloured product which can be determined photometrically. This additional step in the assay generates additional analytical variability. The effect of assay temperature is particularly important: microtitre plates require careful equilibration of the whole plate to avoid temperature differences between inner and outer wells. These 'edge-effects' may significantly affect enzyme activity and lead to large analytical errors. The use of assays reliant upon absorbance measurements tends to limit their working range because, even if a high quality spectrophotometer is used, precise and accurate readings outside the range of 0.05-2.0 absorbance units are difficult to obtain. Therefore, even with high affinity antibodies and optimised assay protocols, the detection system will limit assay performance.

Fluorophores have been employed in many immunoassay systems but interference or quenching effects from other biomolecules have limited their use. However, the development time-resolved fluorimetry in the 1980 s overcame these problems (chapter 1) and this technique, together with chemiluminescence, appears to offer the greatest potential for the development of safe, sensitive and reproducible immunoassays. Both techniques require expensive, specialised instruments for the detection of fluorescence or chemiluminescence which may prohibit their use as a research tool. However, as more commercial assays have become available their use in routine clinical chemistry departments has become widespread: many will now own at least one of the instruments. The availability of a time-resolved fluorimeter in this department provided an opportunity to develop "in-house" time-resolved fluoroimmunoassays.

## **4.2 Lanthanide labelling.**

The theoretical aspects of lanthanide labelling and time-resolved fluorescence measurements have already been described in some detail in the introduction and Material and Methods sections. In this section, various practical aspects of lanthanide labelling are discussed, and antibodies labelled using this technique are characterised.

The initial aim of this project was to produce a set of labelled antibodies to be used (together with non-labelled antibodies) in two-site time-resolved immunoassays for Lp(a). The first antibody to be labelled with europium ( $\text{Eu}^{3+}$ ) was a commercially-available polyclonal anti-apo B antibody obtained from the 'Binding Site', Birmingham, UK. This antibody was chosen because of its availability in affinity-chromatography purified form at a high protein concentration.

Kits for lanthanide labelling were purchased from Wallac. The europium labelling kit contained a glass vial containing 0.2 mg of Europium-DTTA chelate (labelling vial), bottles of enhancement solution, Tris-HCl assay buffer and concentrated wash buffer, a europium standard solution (100 nmol/l), a glass vial of highly purified 7.5% (w/v) bovine serum albumin solution and a 96 well microtitre plate.

The manufacturer's instructions recommend that the labelling procedure be carried out in a different room to where the assay is performed in order to avoid possible contamination of work areas with europium. However, due to space limitations, this was not possible. Nevertheless, every care was taken to ensure that none of the concentrated europium label was spilt onto work surfaces and automatic pipettes used in the labelling procedure were not used in the assay. Europium contamination of the work area is difficult to detect, although as yet there appears to be no evidence (such as increased background counts in the assay) that contamination occurred during any of five labelling procedures.

Before the labelling reaction was initiated, a gel-filtration chromatography column containing Sephadex G-25 and Sephadex 6B was prepared. A 10 ml

disposable plastic pipette (28 cm long with an internal diameter of 0.8 cm) held vertically was partially sealed at the lower end by inserting a plug of porous glass wool. A well mixed slurry of Sepharose 6B was poured slowly into the pipette until a 15 cm column was formed. A suspension of Sephadex G-25 was then used to create an additional 7 cm of column above the Sepharose 6B. A small plastic funnel was attached to the top of the column to act as a fluid reservoir and a 7 cm piece of plastic tubing was attached to the lower tip of the column to aid dispensing of the eluant. Forty ml of elution buffer (see Materials and Methods) was passed through the column which was then sealed at each end until needed. At no time was the column allowed to dry out. It was possible to use the same column on several occasions provided it was first flushed with 50 ml of decontamination buffer (see Materials and Methods) followed by 50 ml of elution buffer to remove any residual europium label or protein.

The coupling reaction between the europium-DTTA chelate and protein is inhibited by compounds containing amine groups, even when they are present at very low concentrations <sup>(180)</sup>. The reaction mixture must therefore be free of buffers containing amine groups, for example, Tris and HEPES. Buffers containing sodium azide should also be avoided. The Binding Site anti-apo B antibody was supplied in a Tris/saline buffer containing 0.1% (w/v) sodium azide. A 'buffer exchange' procedure was therefore employed to separate the antibody from these undesirable compounds. A PD-10 Sephadex chromatography column from Pharmacia was used according to the manufacturer's instructions. 25 ml of europium labelling buffer (see Materials and Methods) was passed through the column. 0.4 ml of the anti-apo B antibody solution containing 6 mg of protein was pipetted onto the column followed by 2.1 ml of labelling buffer and 2.5 ml of eluant was collected. This was shown to be virtually protein-free by its lack of absorbance at 280 nm. A further 3.5 ml of labelling buffer was pipetted onto the column and 3.5 ml of eluant was collected. It was assumed that all 6 mg of protein was present in this fraction. 0.5 ml of this fraction (containing ~0.85 mg of protein) was pipetted into the labelling vial and mixed gently. The

remainder of the solution was stored in aliquots at -20°C to be used in subsequent labelling procedures.

Reaction conditions play a significant part in producing high incorporation of europium into protein. Examples of the effects of labelling buffer pH, and of incubation time and temperature on europium incorporation were supplied by the manufacturer and are reproduced in Figure 4.1. The manufacturer warns that proteins, particularly monoclonal antibodies, may be damaged by exposure to extremes of temperature and pH. Therefore, as a starting point, reaction conditions described by the manufacturers as 'mild' were used for the first labelling. These were : a labelling buffer pH of 8.5, and an overnight (18 hour) incubation at room temperature (20°C).

The entire contents of the labelling vial (i.e. labelled antibody and free label) was then pipetted onto the top of the Sephadex/Sepharose column. The labelling vial was washed with 0.5 ml of elution buffer which was also added to the column. Once the buffer had entered into the column 60 ml of elution buffer was poured into the funnel, and thirty x 2 ml fractions were collected. In subsequent labellings, forty x 0.5 ml fractions were collected. The latter protocol improved resolution between the labelled antibody and free label fractions eluting from the column. The manufacturers recommend that the absorbance at 280 nm of the eluant should be continuously monitored during the separation procedure in order to identify the peak of labelled antibody. Since a suitable automated fraction collector was not available in this department the fractions were collected manually.

Fractions were mixed by vortexing and diluted to 1:10,000 in enhancement solution (see Materials and Methods). 200 µl of the diluted fractions were then pipetted, in duplicate, into the wells of a microtitre plate. The plate was then mixed gently on a plate shaker for 5 minutes before fluorescence measurements were taken on the time-resolved fluorimeter. A plot of fluorescence counts versus fraction number revealed a peak of free europium, a separate peak of monomeric labelled antibody and (on some separations) a peak of aggregated labelled antibody. The

aggregate eluted from the column first, followed by monomeric labelled protein and then the free label. Plots for each labelling procedure are presented as figures 4.2 to 4.7.

This protocol was considered to be adequate to identify the individual peaks obtained, but, as a check, the absorbance of each fraction was measured at 280 nm . A spectrophotometer (Cecil instruments) employing quartz cuvettes was used to read the absorbance of each fraction against a deionised water blank. Peaks for protein content corresponded to the peaks obtained for europium content (figure 4.4). Figure 4.5 also shows an additional peak (likely to be of labelled aggregated antibody) which elutes from the column in the first 4 ml fraction. The labelled protein elutes in the 4-8 ml fraction and the free label elutes in the 8-18 ml fraction.

Fractions known to contain labelled monomeric antibody (those fractions enclosed by the arrows in figures 4.2 to 4.7) were pooled. Care was taken to ensure that aggregated protein was not included in the pool since such protein causes problems of high background and assay imprecision. Approximately 3 ml of labelled antibody solution was obtained from each labelling procedure.

The pooled labelled antibody was then characterised as follows according to the manufacturer's instructions (226):-

### Europium concentration.

The pooled labelled antibody was diluted 1:10,000 and the europium standard was diluted 1:100 in enhancement solution. 200 µl of each solution was pipetted (in quadruplicate) into a microtitre plate which was then shaken gently for five minutes before fluorescence readings were taken. The europium content of the label was calculated as follows:-

$$\frac{\text{counts for diluted label} \times \text{dilution factor (10,000)}}{1000 \times \text{counts of 1 nmol/l europium standard}} \quad (\mu\text{mol/L})$$

A factor of 1000 is used to correct for the conversion of units from nmol/L to µmol/L.

### Protein concentration

The protein concentration (mg/ml) was determined from the absorbance of the pooled labelled antibody solution at 280 nm read against a blank of deionised water. A factor of 0.008 was subtracted from the absorbance for every µmol/L of europium present in the label to compensate for absorbance due to aromatic thiourea bonds formed during the labelling process.

Knowing that the absorptivity value for 1 mg/ml of IgG is 1.34, the protein concentration was calculated as follows :-

$$\frac{(\text{Absorbance at 280 nm}) - (0.008 \times \text{Europium conc } (\mu\text{mol/L}))}{1.34} \quad (\text{mg/ml})$$

To estimate the labelling yield, an approximation of the molar protein concentration was needed. Since the molecular weight of IgG is 160 kDa, the protein concentration is :-

$$\frac{\text{Protein concentration (mg/ml)} \times 1,000,000}{160,000} \quad (\mu\text{mol/L})$$

### Labelling yield.

The labelling yield was calculated by dividing the europium concentration by the protein concentration (both in µmol/L).

The manufacturers recommend that a labelling yield of 5-15 Eu/IgG is desirable although lower yields (2-5 Eu/IgG) should produce satisfactory assays. Yields in excess of 20 Eu/IgG may cause problems due to formation of protein aggregates.

#### Recovery.

An estimate of the percentage recovery of protein initially added to the labelling vial may be made as follows :-

$$\frac{\text{Protein concentration (mg/ml)} \times \text{volume of solution (ml)} \times 100}{\text{Protein added (mg)}}$$

Following these calculations highly purified BSA was added to the labelled antibody pool to a final concentration of 0.1% (w/v). BSA was added as a carrier for the labelled protein and to minimise its adhesion to storage containers. An anti-bacterial agent, sodium azide (0.1%), was already present in the elution buffer. The labelled antibody was divided into 0.5 ml aliquots and stored in plastic containers at 4 °C. Freezing of the label is strongly discouraged by the manufacturers.

### **4.3 RESULTS.**

Three different antibodies were labelled with europium. Two are commercially-available polyclonal antibodies, and the other, the monoclonal antibody (29c10), was generated 'in-house' (described in Chapter 3). The characteristics of each labelled antibody obtained from the labelling procedures is presented in Table 4.1. The elution profile of each of the chromatographic separations is presented in Figures 4.2 to 4.7.

More detail on individual labelling procedures is presented below.



#### **4.3.1 'Binding Site' anti-apo B polyclonal antibody.**

This antibody was labelled on three occasions (figs 4.2 to 4.4). The first two labellings used antibody that had just undergone the buffer exchange procedure while the third labelling used antibody that had undergone buffer exchange for labelling on 5/10/92 and was then stored for 11 months at -20°C until use.

Only in the third labelling procedure was the protein content (determined by absorbance 280 nm) estimated together with the europium content. This data (shown in figure 4.4) confirmed that the peaks identified by estimation of europium content were the same as those that contained the labelled antibody.

Data in table 4.1 shows that, providing reaction conditions were kept reasonably constant, the europium yield remained constant. Increasing the labelling time (labelling 3 on 2/9/93) by 6 hours elicited a proportionate increase in europium incorporation (8.7-11.7 europium atoms/antibody).

#### **4.3.2 'Immuno' anti-apo (a) polyclonal antibody.**

This antibody, which was labelled on a single occasion (fig 4.5), gave rise to the most discrete separation of fractions. In addition, a relatively large peak containing aggregated labelled antibody was also seen, although this peak was not included in the labelled antibody pool.

#### **4.3.3 29c10 anti-apo (a) monoclonal antibody.**

This antibody was labelled on one occasion but with a slightly different labelling protocol: the pH of the labelling buffer was increased from 8.5 to 8.65 in an attempt to improve europium incorporation into the antibody. However, the resulting label had the lowest europium incorporation of all (figs 4.6 & 4.7)! At 0.2 Eu atoms/antibody, it was considered that this label would not produce a satisfactory assay. Surprisingly, even with such a low europium content, the label was found to be suitable for use in Lp(a) assays (chapter 5).

#### 4.4 DISCUSSION.

These results demonstrate that the labelling of antibodies with europium is a simple procedure requiring a minimum of specialised apparatus. There are no known safety hazards associated with the procedure and, providing meticulous care is taken to avoid contamination of work areas, the assay can be performed in the same room as the labelling procedure.

Since individual antibodies exhibit differing degrees of europium incorporation during labelling, optimisation of the reaction conditions should be performed for each labelled protein. Ideally each parameter of the labelling procedure (reaction mixture pH, temperature and incubation time) should be optimised to maximise labelling yield. However, the cost of the kit is a major limiting factor and such extreme optimisation would probably cost in excess of £1000 for each antibody. The relatively 'mild' labelling conditions (short incubation at low pH and 20°C) which were used in this project appeared to give satisfactory yields for each polyclonal antibody that was labelled. However, labelling yield was much lower for the monoclonal antibody.

The reason for the very low europium incorporation into the 29c10 antibody is unclear. Since the Eu-DTTA chelate reacts with amino groups on protein, it is possible (although unlikely) that the 29c10 antibody contains relatively few of these groups. Other possibilities include contamination of reagents with amine-containing compounds, or the reaction conditions used were too mild for adequate incorporation. Increasing the reaction mixture pH to 8.65 was very unlikely to have damaged the 29c10 antibody, since this antibody is stable in the bicarbonate buffer (pH 9.2) used for antibody coating of microtitre plates (see chapter 5).

One of the great advantages of lanthanide labels is their stability. Storage of labelled antibodies at 4°C in 0.5 ml aliquots is recommended. Although no formal stability studies have been made there appeared to be no loss in assay performance when labels were stored for up to one year. As a precautionary measure, the antibody is passed through a 0.22µm filter before use in an assay to remove any aggregates of

labelled antibody which occasionally form since such aggregates have been shown to adversely effect assay precision (data not shown).

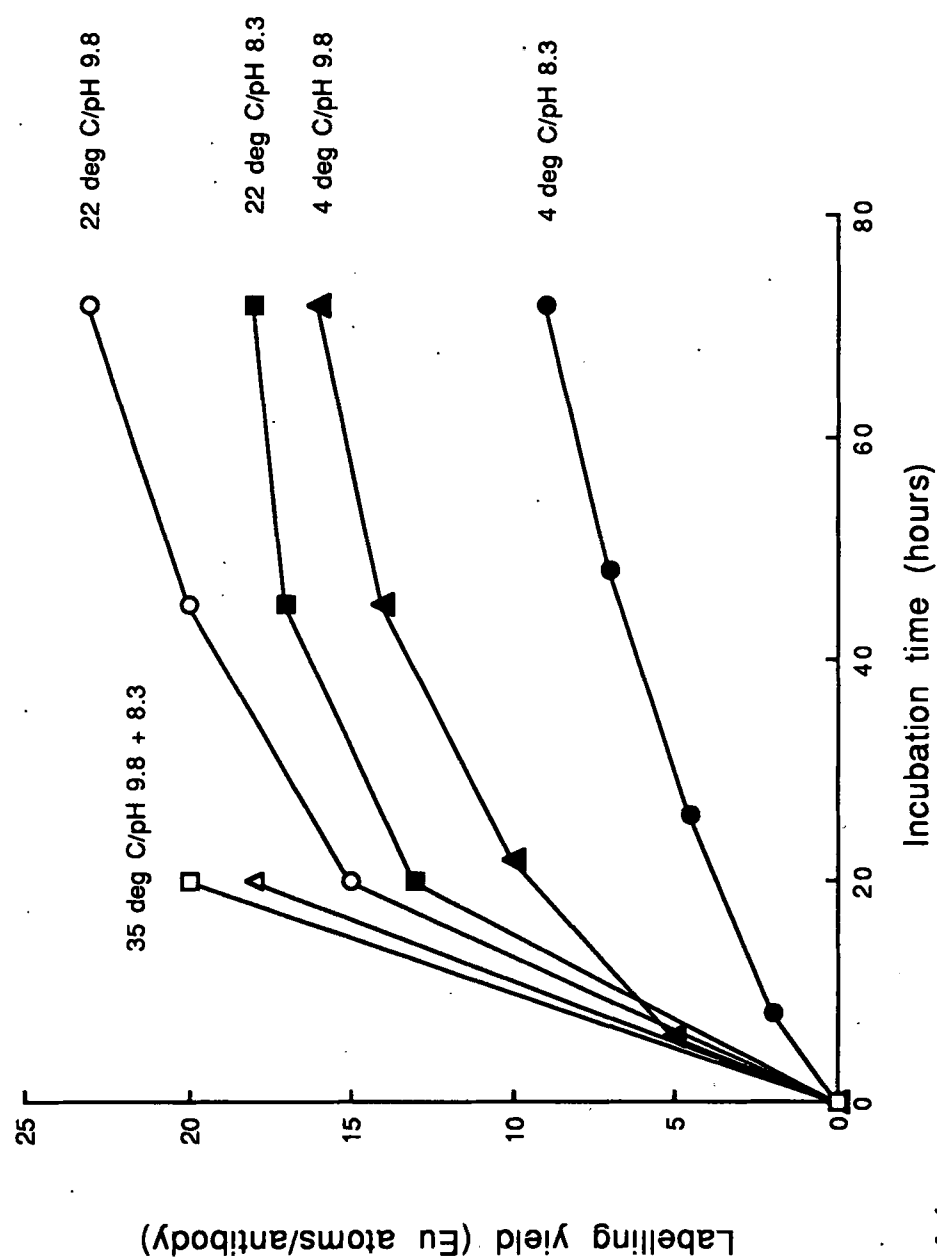
A labelling kit using another lanthanide, samarium, is also commercially available. Although the intensity of emitted fluorescence from samarium is less than for europium, the use of antibodies labelled with either of these two lanthanides offers the prospect of dual-analyte assays on a single microtitre plate.

In conclusion, the labelling of antibodies with europium is an excellent alternative to conventional radioisotope or enzyme labelling. The cost of reagents is not high considering that one labelling procedure may produce sufficient antibody for over 100 microtitre plate assays. Importantly, there are no requirements for special working environments and no known safety hazards.

**TABLE 4.1 CHARACTERISATION OF LABELLED ANTIBODIES.**

The europium and protein concentrations of five labelled antibody solutions.  
All labellings were performed at room temperature (~20°C) and pH 8.5.

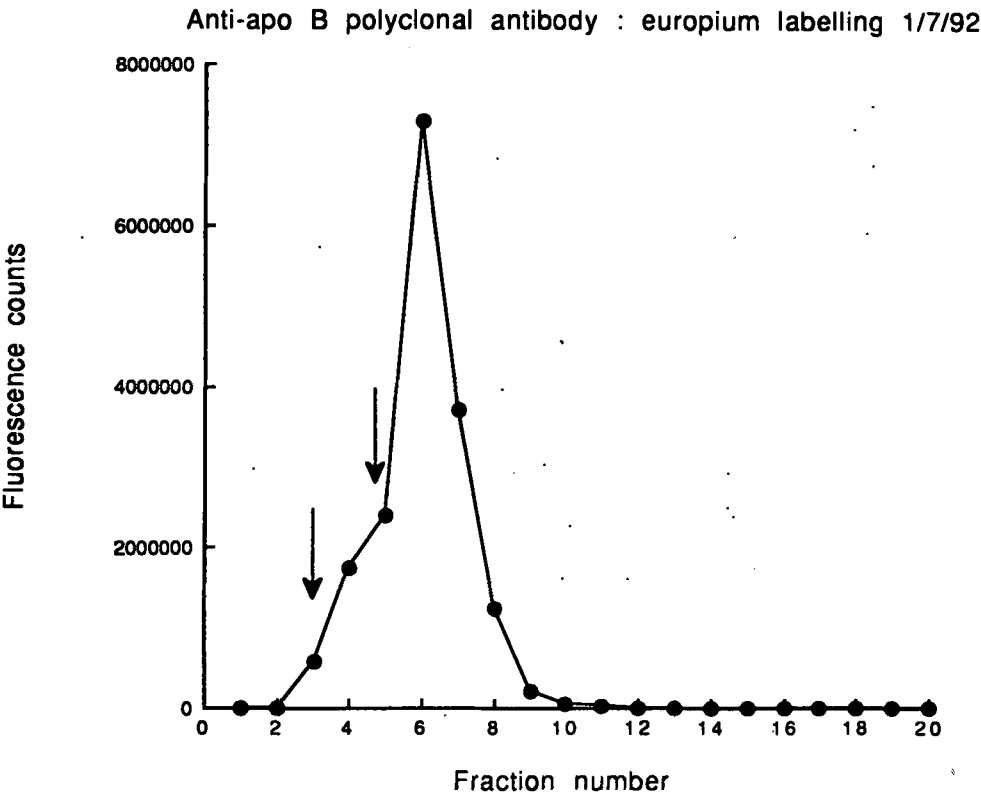
Date	Antibody	Europium concentration (μmol/l)	Protein concentration (mg/ml)	Europium yield (Eu/Antibody)	Protein added (mg/0.5 ml)	Protein recovery (%)	Incubation time (hours)
1/7/92	anti-apo B	6.4	0.14	7.3	0.85	95	18
5/10/92	anti-apo B	6.8	0.125	8.7	1.25	80	18
29/9/93	anti-apo B	24.6	0.336	11.7	1.25	102	24
30/11/92	anti-apo (a)	19.2	0.469	6.6	0.98	101	18
31/3/93	29c10	0.48	0.336	0.23	1.03	91	19



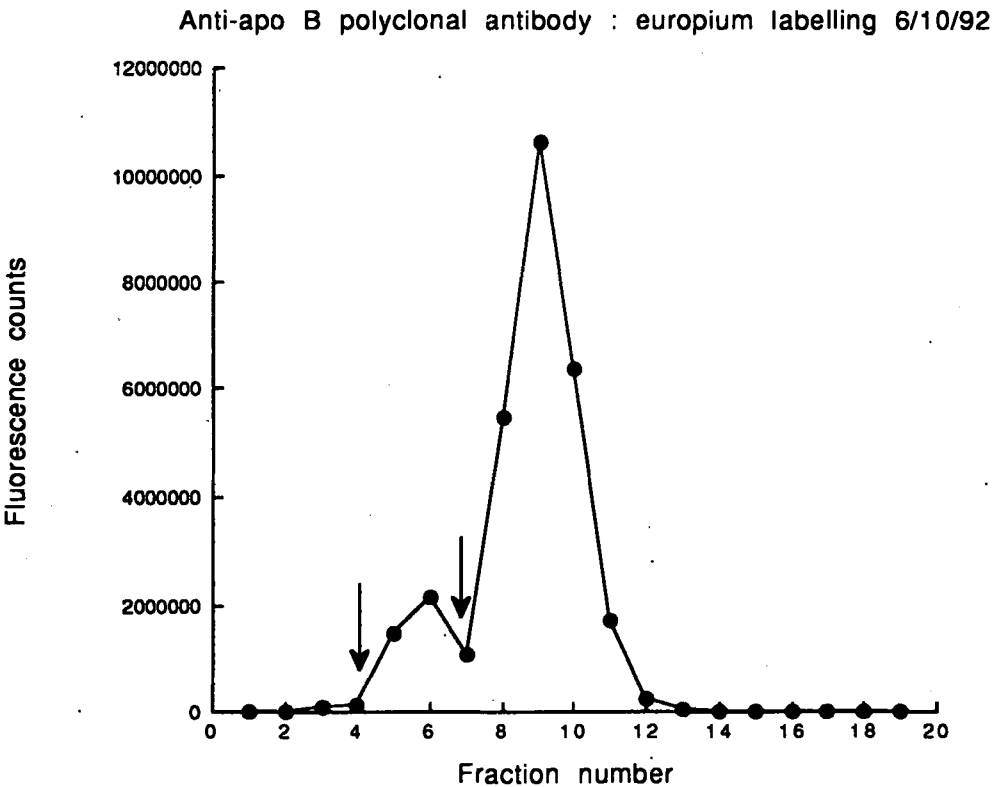
**FIGURE 4.1**

The effects of incubation time, buffer pH and temperature on europium incorporation into proteins. Reproduced from the DELFIA europium labelling kit instruction booklet.

**FIGURE 4.2**



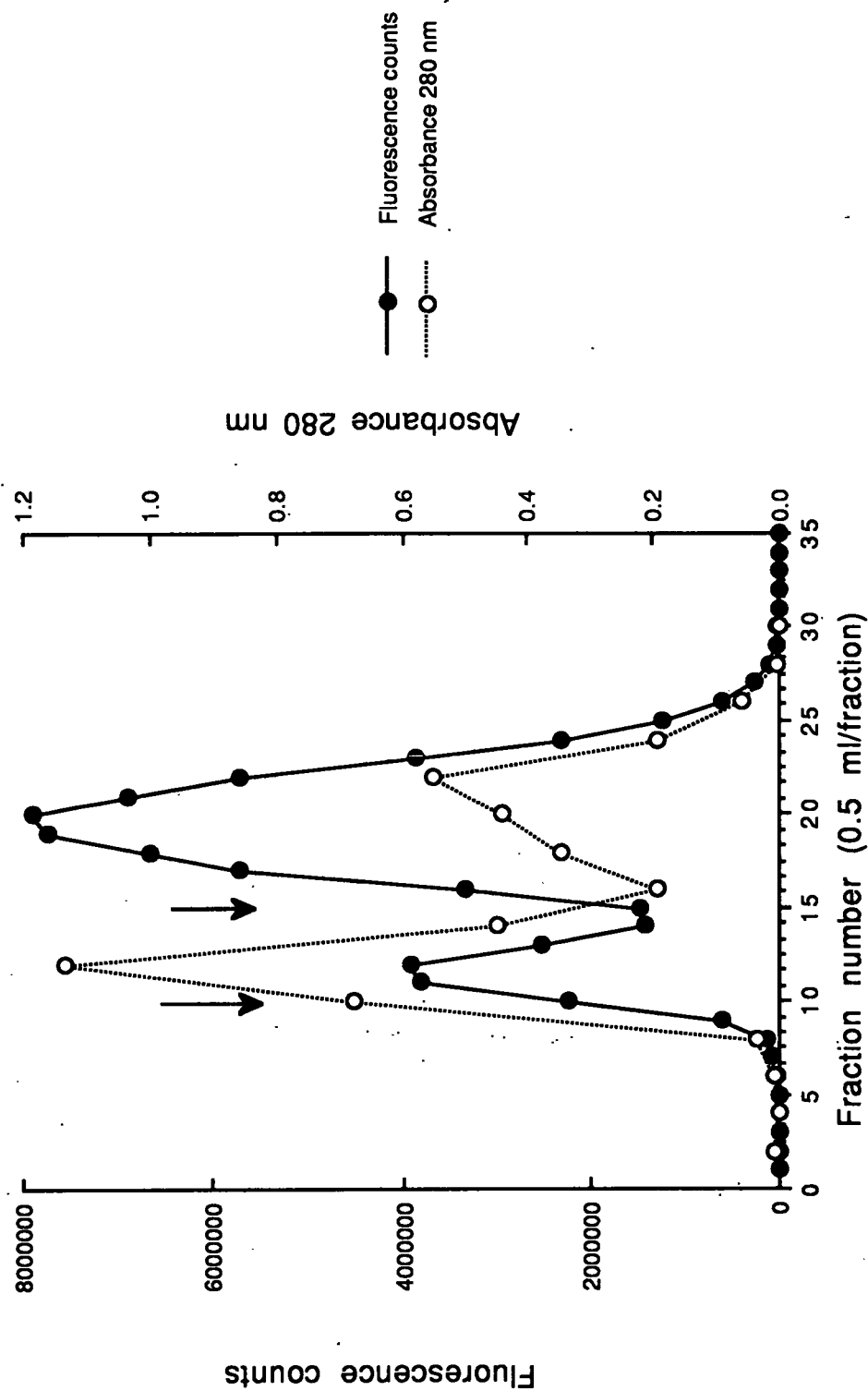
**FIGURE 4.3**



Elution profiles for two europium labellings of an anti-apo B polyclonal antibody. Fractions between the arrows were pooled to make the working labelled antibody.

**FIGURE 4.4**

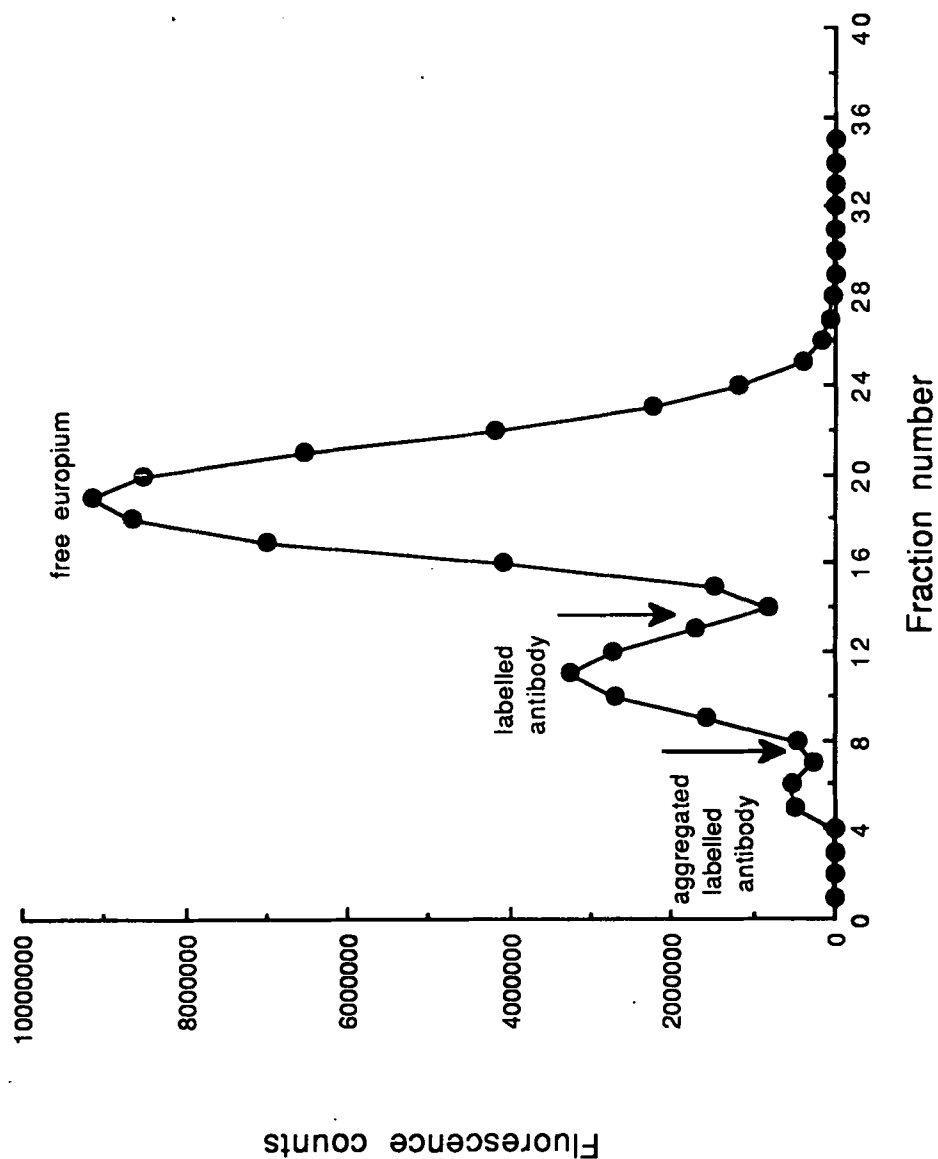
Anti-apo B polyclonal antibody : europium labelling 29/9/93



Elution profile for the europium labelling of an anti-apo B polyclonal antibody.  
Europium and protein content (proportional to absorbance at 280 nm) of the fractions.

**FIGURE 4.5**

Immuno anti-apo(a) polyclonal antibody : europium labelling 30.11.92

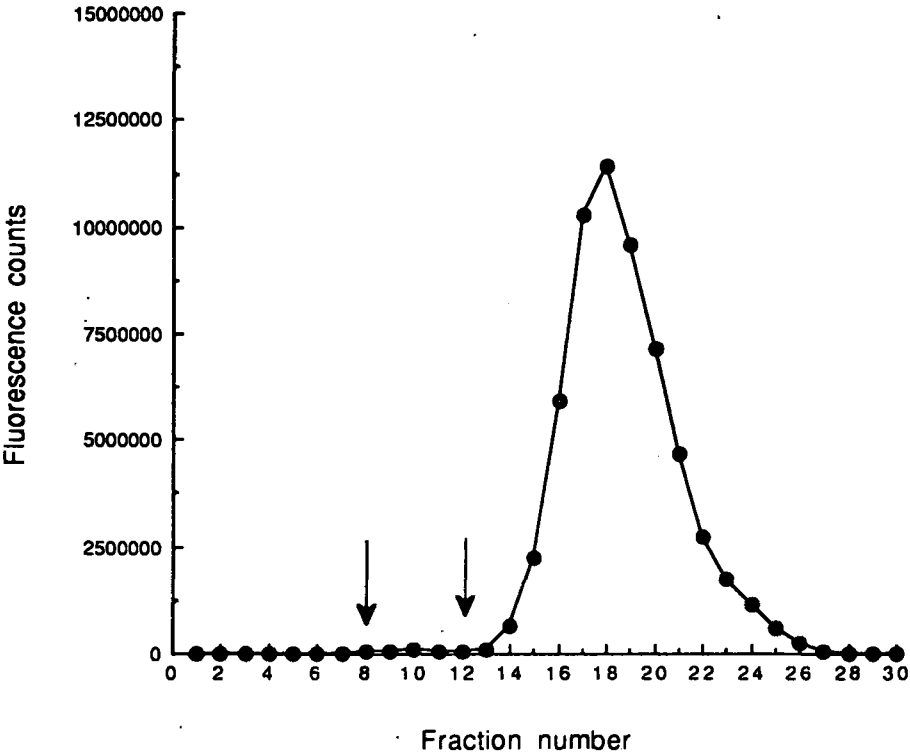


Elution profile for the labelling of an anti-apo (a) polyclonal antibody.  
Fractions between the arrows were pooled to produce the working labelled antibody.



**FIGURE 4.6**

29C10 anti-apo (a) monoclonal antibody : europium labelling 31/3/93



**FIGURE 4.7**

29C10 anti-apo (a) monoclonal antibody : europium labelling 31/3/93

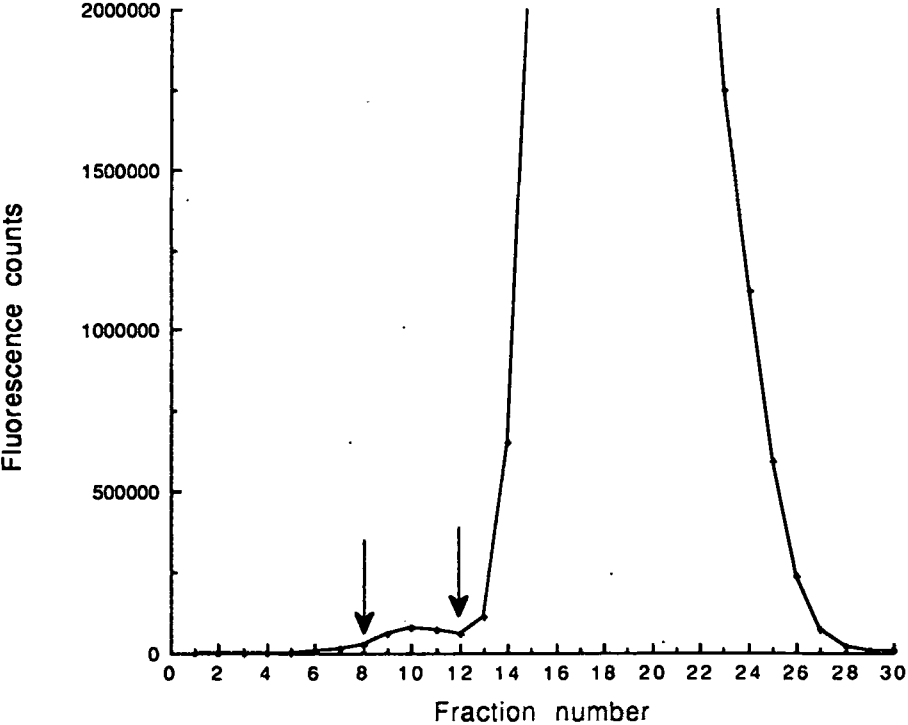


Figure 4.6 : an elution profile for labelling of an anti-apo (a) monoclonal antibody, 29c10. Figure 4.7 shows an enlargement of figure 4.6. Fractions between the arrows were pooled to make the working label.

**CHAPTER 5**  
**DEVELOPMENT AND OPTIMISATION**  
**OF A TIME-RESOLVED FLUOROIMMUNOASSAY FOR Lp(a)**

## **5.1 Introduction.**

Optimisation of each phase of an immunoassay is essential to ensure reliable analytical performance. Data obtained during experiments designed to optimise each step of the time-resolved fluoroimmunoassay for Lp(a) are presented in this chapter.

The following assay protocol was used (unless stated otherwise):-

- (i) Microtitre plates were coated with an anti-Lp(a) capture antibody.
- (ii) The plates were washed to remove unbound capture antibody.
- (iii) Binding sites on the wells were 'blocked' using a solution of bovine serum albumin (BSA).
- (iv) Excess unbound BSA was removed by washing with a Tris/HCl wash buffer.
- (v) Diluted sample was added to the well to allow binding of Lp(a) in the sample to the capture antibody.
- (vi) The microtitre plate was washed to remove unbound diluted sample.
- (vii) Europium -labelled anti-Lp(a) detection antibody was added to each well
- (viii) The plates were washed to remove unbound europium-labelled antibody.
- (ix) Enhancement solution was added to each well. The resulting fluorescence was detected and measured in each well.

Suitable incubation times were employed for each of the steps.

## **5.2 Coating of microtitre plates with antibody.**

### **5.2.1 Introduction.**

Development of a two-site microtitre plate-based immunoassay requires one of the antibodies to be immobilised on the plate. This may be achieved by a variety of methods (227). The simplest involves a process whereby the antibody, in alkaline solution, passively adsorbs to the plastic microtitre well. The technique does not entail the formation of any chemical bonds and results in little damage to the protein structure (227). The process is sometimes known as 'passive diffusion' (224). Most assays using this method of microtitre plate coating involve dilution of the antibody in bicarbonate buffer (pH~9.0) and overnight incubation at 4°C.

Development of an assay for Lp(a) presented a theoretical problem of specificity: since the plate coating (capture) antibody was exposed to serum containing apo B (present in particles other than Lp(a)) <sup>(228)</sup> as well as apo (a) (present only in Lp(a)) the use of an anti-apo B antibody to capture Lp(a) might allow competition for antibody binding sites between apo B present in Lp(a) and apo B present in the other serum lipoprotein particles. In contrast, the use of an anti-apo (a) capture antibody would present no competition problems because apo (a) is present only in Lp(a) <sup>(33)</sup>. For this reason it was decided to use an anti-apo (a) capture antibody. Three anti-apo (a) antibodies were available: a commercial polyclonal antibody from 'Immuno' and two monoclonal antibodies (29c10 and 35f5) which were produced in-house. The performance of each of these as capture antibodies was investigated.

### **5.2.2 Optimising the antibody coating of microtitre plates.**

#### **(i) Choice of antibody dilution.**

Each antibody was diluted in bicarbonate buffer (pH 9.2) to produce a range of concentrations which were used to coat microtitre plates (250µl of each solution per well overnight at 4°C). For each antibody, a serum sample containing an elevated concentration of Lp(a) (~800 mg/L) was assayed using the standard protocol described above. A plot of fluorescence counts versus antibody dilution was used to determine optimal antibody dilution (i.e. the antibody dilution at which small changes in dilution had little effect on the number of fluorescence counts).

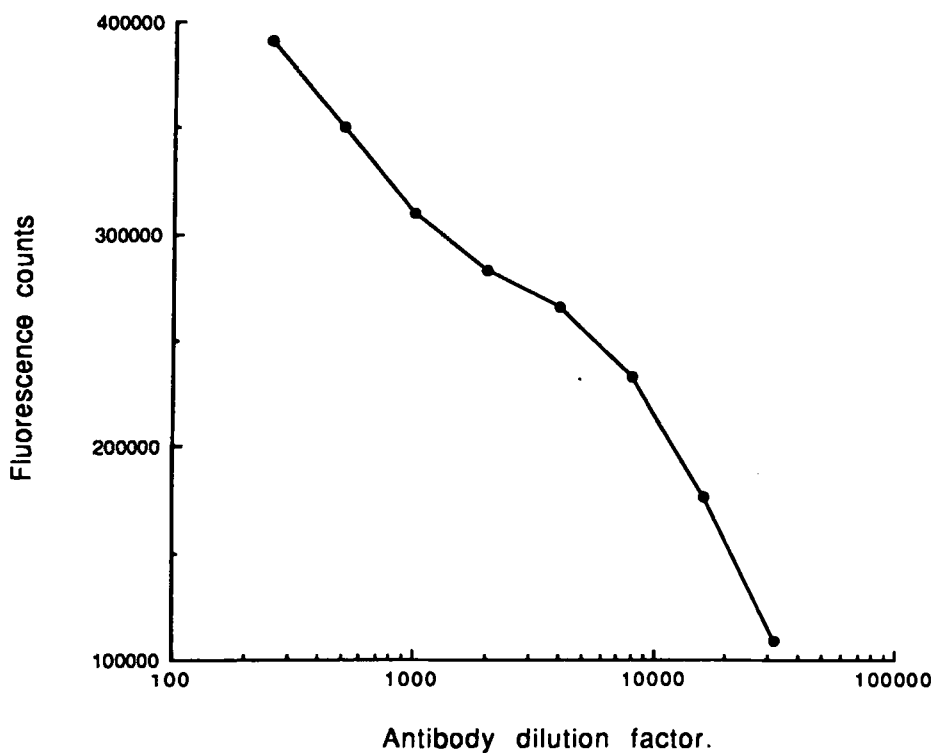
*'Immuno' anti-apo (a) polyclonal antibody* (~6 mg/ml) was diluted 1:250 in bicarbonate buffer and serially diluted 1:2 to give a final dilution of 1:32000. These solutions were then used to coat microtitre plates. A steady increase in fluorescence counts was observed with decreasing antibody dilution (figure 5.1). This suggests that maximum binding of capture antibody to the microtitre plate did not occur with

the antibody dilutions used. A (sub-optimal) dilution of 1:250 was chosen to coat subsequent plates in order to conserve antibody stocks.

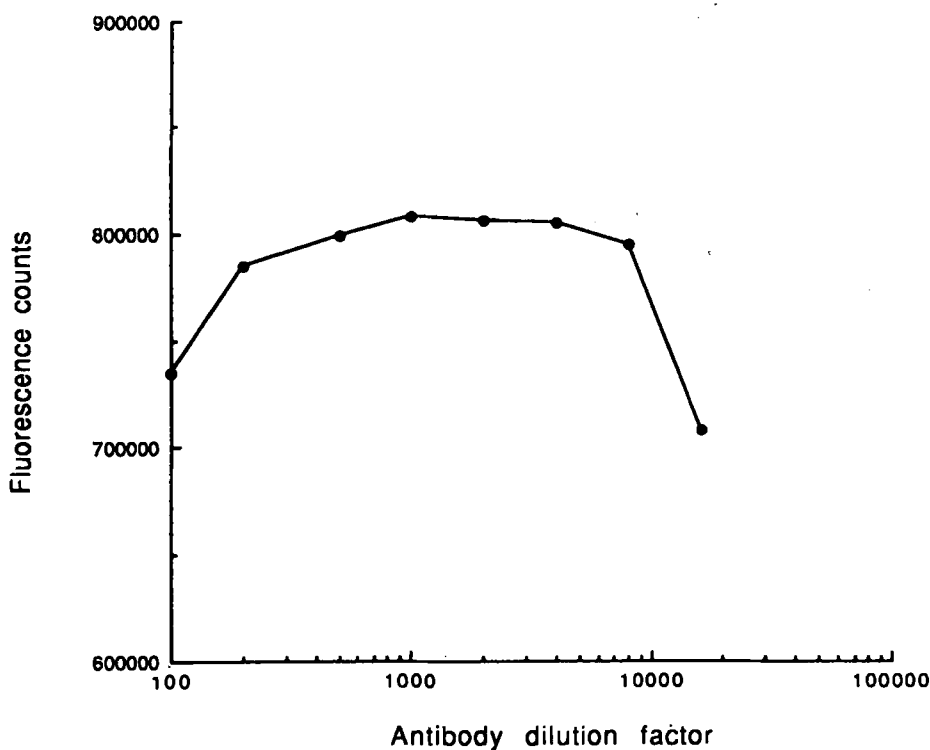
*35f5 antibody* was diluted directly from the ascitic fluid (without purification) to 1:125 - 1:16000 and used to coat microtitre plates. Since the antibody concentration of the ascitic fluid was not known, it was not possible to calculate the amount of antibody added per well.

The plot of fluorescence counts versus antibody dilution are shown in figure 5.2. Unexpectedly, fluorescence counts were lower at both high and low dilutions of the antibody. The reduction of counts at high antibody dilution may have resulted from the antibody being present in limiting amount (i.e. fewer antibody molecules coating the plate than Lp(a) molecules present in the sample). However, the reason for the decrease in fluorescence counts at low antibody dilution is less clear. Possibly the presence of relatively large quantities of other proteins (such as albumin) in the ascitic fluid may block the non-specific adsorption of the antibody to the plastic microtitre plate. To eliminate experimental error the experiment was repeated on a further two occasions with identical results. A dilution of 1:1000 was chosen for assay development, this value being at the plateau of the plot (figure 5.2).

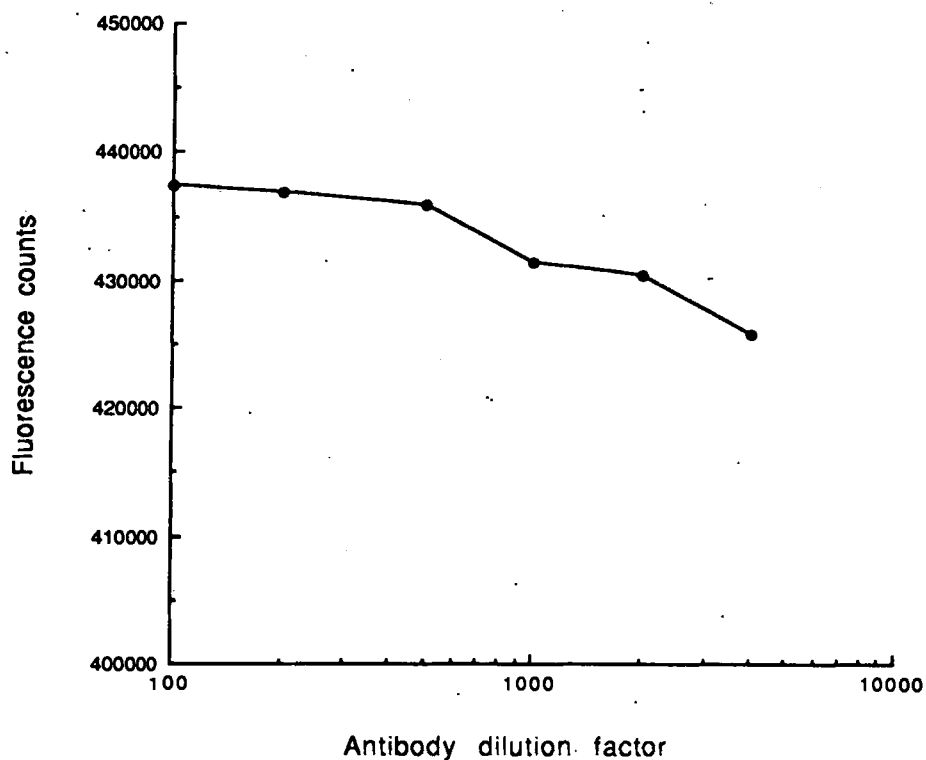
*29c10 antibody* (7.2 mg/ml - purified by hydroxyapatite adsorption chromatography) was diluted 1:125 and serially diluted 1:2 to give a final dilution of 1:4000: these dilutions were used to coat microtitre plates. Fluorescence counts at each antibody dilution are shown in figure 5.3. It is apparent that a 1:1000 antibody dilution (although slightly sub-optimal) would be suitable for assay development because small changes in antibody dilution have little effect on fluorescence counts.



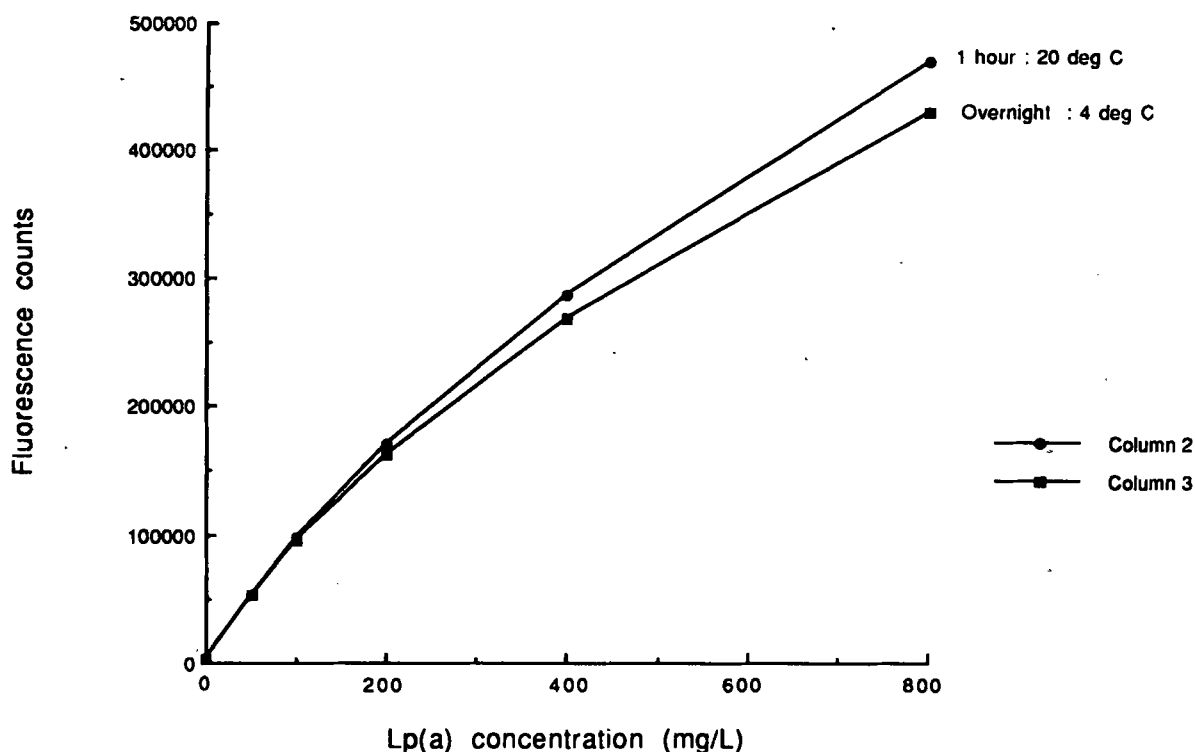
**Figure 5.1** An antibody dilution curve for the 'Immuno' anti-apo (a) capture antibody. Fluorescence counts for an 800 mg/L Lp(a) standard vs dilution of capture antibody. Europium-labelled anti-apo B detection antibody



**Figure 5.2** An antibody dilution curve for the 35f5 capture antibody. Fluorescence counts for an 800 mg/L Lp(a) standard vs capture antibody dilution. Europium-labelled anti-apo B detection antibody



**Figure 5.3** An antibody dilution curve for 29c10 capture antibody.  
Fluorescence counts for an 800 mg/L Lp(a) standard vs capture antibody dilution.  
Europium-labelled anti-apo B detection antibody



**Figure 5.4** A comparison Lp(a) standard curves assayed on microtitre plates coated with anti-apo (a) antibody overnight at 4 deg C or for 1 hour at 20 deg C.  
Europium-labelled anti-apo B detection antibody

## **(ii) Plate coating incubation time.**

Antibody coating of microtitre plates is usually performed during overnight incubation at 4°C <sup>(227)</sup>. An experiment was performed to determine the minimum time necessary to achieve adequate coating with anti-apo (a) antisera without deterioration in assay performance. Microtitre plates were coated either overnight at 4°C or 1 hour at 20°C. To assess the efficiency of the antibody coating, a series of Lp(a) standards were analysed using the standard assay protocol. The standard curves thus obtained are shown in figure 5.4. There appears to be little difference between the curves obtained at each temperature, suggesting that microtitre plate coating with this antibody was complete within one hour. An experiment using incubation times of less than one hour at 20°C suggested that antibody coating was virtually complete after 20 minutes (data not shown). The microtitre plate coating properties of the monoclonal antibodies, 29c10 and 35f5 were also investigated: the data obtained (not shown) verified that a 1 hour incubation at 20°C was sufficient for optimal antibody coating of microtitre plates. It was decided that 1 hour would be the minimum time used for antibody coating since shorter incubation times might lead to inconsistent coating, thereby causing imprecision in the assay.

## **(iii) Re-use of coating solution.**

Reports suggest <sup>(227)</sup> that only a small proportion of the antibody in coating solutions actually binds to the plastic of the microtitre plates. It would be wasteful to discard the antibody solution following its incubation with the microtitre plates. Indeed, in principle there is no objection to the re-use of this solution to coat additional plates. Plates were coated with each of the three anti-apo (a) antibodies at their optimal dilutions. After coating, the solutions were collected and used to coat another microtitre plate. This procedure was repeated up to 10 times. Approximately 95% of the antibody coating solutions was recycled and used for the next coating procedure; the solutions were stored at 4°C when not in use. A series of Lp(a) standards were assayed using microtitre plates coated either with freshly diluted



antibody or with 'recycled' antibody solution (of the same initial antibody concentration) that had already been used to coat 10 microtitre plates. Standard curves produced using microtitre plates coated with each of the three anti-apo (a) antibodies are shown in figures 5.5 - 5.7. It is apparent that there are only slight differences in each pair of curves. Hence, as it is highly unlikely that these slight changes would significantly affect assay performance, antibody solutions were re-used for up to ten occasions throughout this study.

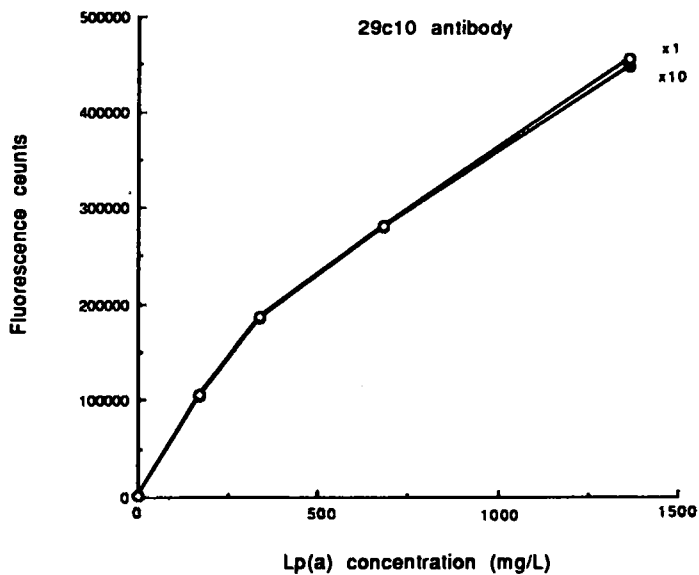
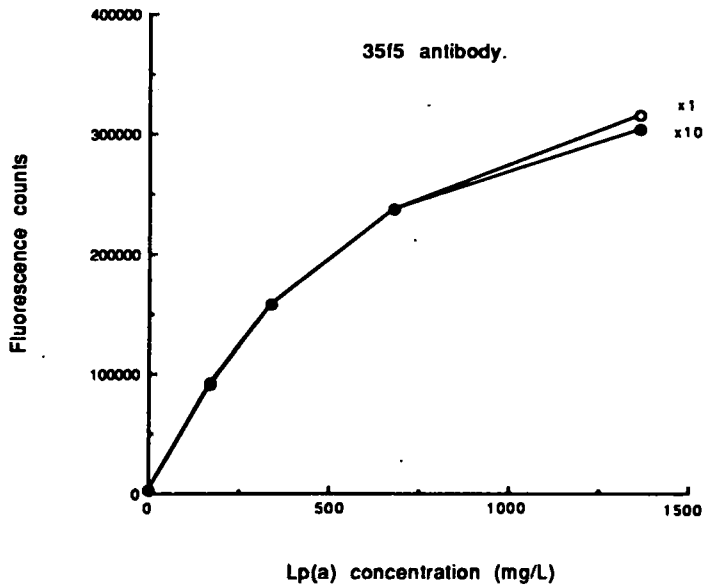
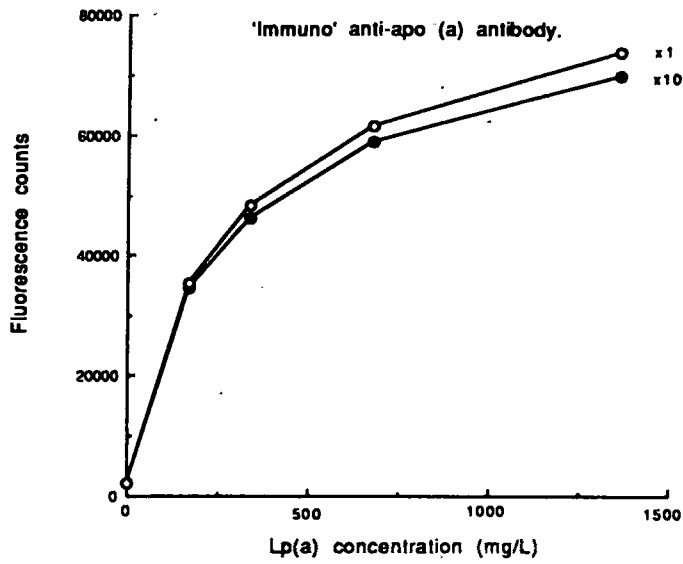
#### **(iv) Stability and storage of coated plates.**

One of the major variables in the performance of microtitre plate assays is inconsistent coating of antibody to the plate <sup>(229)</sup>. This variability may be minimised by preparing a large batch of microtitre plates (using the same coating antibody solution) to be stored prior to subsequent use. Microtitre plates were coated with antibody, blocked with BSA (section 5.3) and stored desiccated for up to 90 days at 4°C. After storage, the efficiency of the antibody coating was assessed by analysing a series of Lp(a) standards using the stored plates and comparing the results with those obtained using plates freshly coated with antibody. The standard curves thus obtained (not shown) were virtually superimposable, suggesting that antibody coated microtitre plates may be stored for three months at 4°C with no loss in assay performance. However, when an antibody coated plate was stored for six months under these conditions some loss in performance, particularly precision of duplicates, was seen (data not shown).

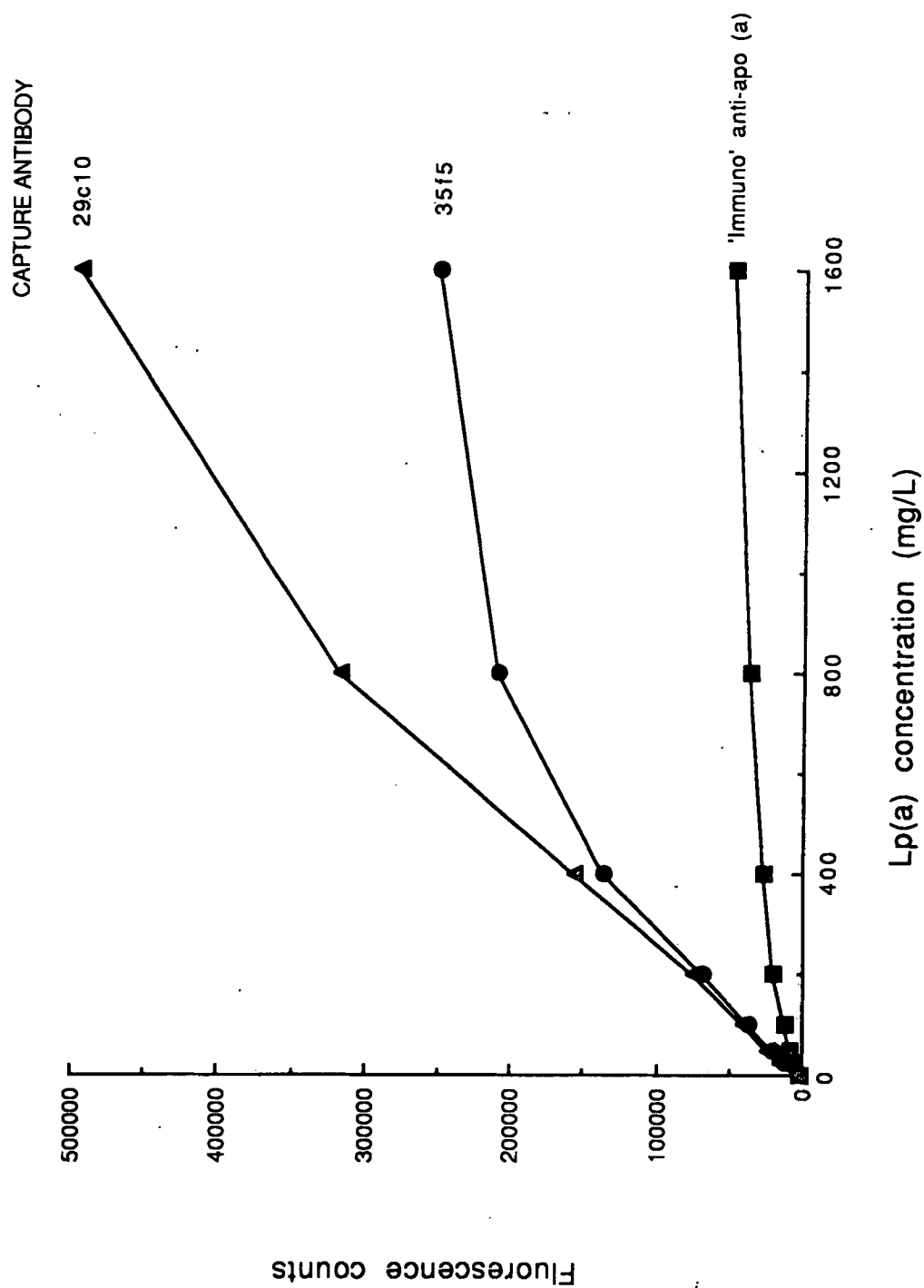
Each of the antibodies were found to be stable when used to coat microtitre plates. However, when a series of Lp(a) standards were analysed on microtitre plates coated with each of the three antibodies at their 'optimum' conditions (figure 5.8) it is apparent that the most linear dose-response curve and highest fluorescence counts were obtained using plates coated with 29c10. Therefore, the 29c10 antibody was the most suitable for coating microtitre plates.

**Figures 5.5-5.7**

Comparisons of Lp(a) standard curves generated in microtitre plates coated with freshly diluted anti-apo (a) antibodies (x1) or anti-apo (a) antibodies used to coat microtitre plates on 10 occasions (x10).



Each assay used the Europium-labelled anti-apo B detection antibody



**Figure 5.8** Comparison Lp(a) standard curves assayed using three different anti-apo (a) capture antibodies under optimal conditions.

Europium-labelled anti-apo B detection antibody

### **5.3 Blocking of non-specific binding sites.**

Most microtitre plate assays use bovine serum albumin (BSA) to occupy ('block') sites on the microtitre plate wells that might otherwise bind assay reagents non-specifically (i.e. at low affinity) <sup>(230)</sup>. In a two-site immunoassay, non-specific binding is expressed as the signal obtained for the zero standard, which should (ideally) be close to zero. A low and precise signal for the zero standard is essential for good assay sensitivity <sup>(231)</sup>. A 400 g/L solution of BSA was prepared in PBS, stored at 4°C, and diluted for use as required.

#### **5.3.1 Volume of 'blocking' solution.**

It is standard procedure that the volume of BSA solution used to block microtitre plate wells should exceed the largest reaction volume used <sup>(230)</sup>. For instance, a reaction volume of 200µl requires a minimum blocking volume of 300µl, particularly if the reaction mixture is to be shaken during incubation (the vortex thus created causing liquid to rise to a higher level in the wells than when at rest). The effect of using different volumes of blocking solution was investigated using microtitre plate wells coated with 29c10 antibody which were 'blocked' with either 100, 250, 300 or 400µl of BSA solution (1% w/v). The plate was 'blocked' for 1 hour at 20°C and, after washing away unbound BSA, fifteen replicates of an Lp(a) zero standard (assay buffer) were assayed for each volume of BSA. The mean and standard deviation (SD) of the fluorescence counts for these wells (presented in table 5.1) give an indication of the efficiency of the 'blocking' : a low mean and SD reflecting efficient blocking.

**Table 5.1.**

Mean/Standard deviation fluorescence counts for 15 replicates of a Lp(a) zero standard assayed using the 29c10-B time-resolved fluorescence Lp(a) assay on microtitre plates 'blocked' with 100-400  $\mu$ l BSA (1% w/v)

Volume of 1% (w/v) BSA				
Fluorescence counts	100 $\mu$ l	250 $\mu$ l	300 $\mu$ l	400 $\mu$ l
mean	8579	3105	1536	1570
SD	6542	356	85	102

It is apparent that blocking with a low volume of BSA is inadequate since very high and imprecise fluorescence readings are obtained. At least 300 $\mu$ l of BSA is required for efficient blocking. The fact that 250 $\mu$ l of BSA is insufficient for complete blocking of the plate suggests that a reaction volume of 200 $\mu$ l rises during shaking above the level occupied by 250 $\mu$ l of solution at rest. 300 $\mu$ l of 1% (w/v) BSA solution was therefore used during assay development.

### **5.3.2 Concentration of 'blocking' solution.**

1% (w/v) BSA is commonly employed as a plate blocking agent in immunoassays <sup>(230)</sup>. Microtitre plates 'blocked' with this solution were compared with those 'blocked' with a more concentrated (5% w/v) solution to determine whether 'blocking' efficiency could be improved. Fifteen replicates of zero standard were assayed using a 29c10 coated microtitre plate that had been 'blocked' with 300 $\mu$ l of either 1 or 5% (w/v) BSA for 1 hour at 20°C. The mean and SD of the fluorescence counts are presented in table 5.2.

**Table 5.2.**

Mean/Standard deviation fluorescence counts for 15 replicates of a Lp(a) zero standard assayed using the 29c10-B time-resolved fluorescence Lp(a) assay on microtitre plates 'blocked' with 300 µl BSA (1 or 5 % w/v)

Fluorescence counts	BSA concentration (% w/v)	
	1	5
mean	1536	1596
standard deviation	85	83

It is apparent that increasing the concentration of BSA has little effect on precision or number of counts for the zero standard.

Increasing BSA blocking times from 1 hour at 20°C up to 5 days at 4°C was shown to had no adverse effects on Lp(a) assay performance (data not shown).

#### **5.4 Washing of microtitre plates.**

Adequate washing of microtitre plate-based immunoassays is essential for good precision and low background counts <sup>(232)</sup>. In this study, microtitre plates were washed with a diluted Tris-HCl buffer supplied with commercial DELFIA® kits. Washing was performed using a DELFIA® automated plate washer. Up to 9 wash cycles (each dispensing 400µl of wash buffer) are possible using this instrument, and after the final wash cycle the plate is aspirated to near-dryness.

A suitable number of wash cycles were arbitrarily selected for each step of the Lp(a) assay as follows:

Wash following coating of microtitre plate with antibody .	3 cycles
Wash following BSA 'blocking' of microtitre plate.	1 cycle
Wash following incubation with diluted sample.	4 cycles
Wash following incubation with labelled antibody.	6 cycles

To assess the efficiency of the washing, europium labelled antibody was added to 16 wells 'blocked' with BSA. It was estimated that the europium added to

each well would produce ~170,000,000 fluorescence counts. The plate was washed for 6 cycles after the addition of labelled antibody and the fluorescence remaining in the wells measured. Using this procedure, less than 2000 counts remained in each well, which represented 0.001% of the total counts added. The selected washing procedure is therefore highly efficient.

### **5.5 Optimising the assay protocol.**

In immunoassays the incubation of sample analyte with capture and detection antibodies may be performed either as a single step (in which sample, capture and detection antibodies are incubated together) or in two sequential steps (in which, following incubation of the sample with capture antibody, unbound sample is removed by washing and detection antibody is then added) <sup>(233)</sup>.

Of the four antibodies suitable for use in the Lp(a) assay, three are directed against epitopes on apo (a), and the other to an epitope on apo B. The anti-apo B antibody and two of the anti-apo (a) antibodies were labelled with europium. For the reasons discussed in section 5.2.1 it was decided that the anti-apo B antibody could not be used to coat the plate. Therefore, assays were developed using this antibody as a detection antibody after Lp(a) was immobilised using an anti-apo (a) antibody. This antibody combination ensured that interference from other apo B-containing lipoproteins in human serum and from plasminogen was eliminated. The use of an anti-apo (a) antibody to capture Lp(a) minimised potential effects of variable binding of the different Lp(a) isoforms. It is likely that these effects would be greater when Lp(a) bound to a detection antibody rather than a capture antibody for the following reasons: the Lp(a) particle needs only to be bound at one site on a capture antibody to become immobilised on a microtitre plate, therefore, binding at more than one site (i.e. if the capture antibody recognises the kringle 4 repeat domain of Lp(a)) should not increase the amount of Lp(a) bound to the microtitre plate. However, if the binding of the detection antibody is influenced by isoform size, then for one isoform

(of low molecular weight) it may be that only one labelled antibody molecule will bind while for another isoform (of high molecular weight) more than one labelled antibody may bind. This effect would obviously lead to spurious assay results.

#### **5.5.1 Assays using an anti-apolipoprotein B detection antibody; (a)-B; 29c10-B; 35f5-B.**

A series of Lp(a) standards (diluted 1:500) were assayed using anti-apo (a) coated microtitre plates employing either a two-step or a one-step protocol. Equal concentrations of sample and detection antibody were used in each case and the total incubation time of each assay was identical (5 hours at 20°C).

Figure 5.9 shows the standard curves obtained using each protocol, the one-step assay produces a sharp increase in fluorescence counts (compared with the two-step protocol) at low analyte concentrations which then decreases at higher Lp(a) concentrations. In contrast, the two-step assay produces a steady increase in fluorescence counts with increasing analyte concentration across the assay range. A repeat of this experiment using a 1:3000 dilution of the Lp(a) standard produced similarly shaped standard curves (data not shown). The non-linearity encountered in the one-step assay may be attributed to the presence of other apo B-containing proteins in the Lp(a) standard (which is derived from pooled human serum). These proteins, present in greater quantity than Lp(a) is in normal serum (1-2 g/L), may compete with Lp(a) for the europium labelled anti-apo B detection antibody. In the Lp(a) standard at 1:500 dilution there is a high concentration of non-Lp(a), apo B-containing proteins, relative to Lp(a). Therefore, the majority of labelled antibody is bound to these proteins in preference to Lp(a). Consequently, the fluorescence counts for standards with a high Lp(a) concentration are reduced. However, when the standard was diluted further (to 100 mg/L) the total concentration of apo B-containing proteins was considerably lower and competition between apo B in Lp(a) and other proteins for the labelled antibody is minimised. The addition of higher concentrations



of labelled antibody to the one-step assay improved linearity, but also increased non-specific binding (data not shown).

### **5.5.2 Assays using anti-apolipoprotein (a) detection antibodies.**

In principle, the use of an anti-apo (a) detection antibody to both capture and detect Lp(a) should eliminate problems due to the presence of apo B in serum samples. However, the structural homology of apo (a) with plasminogen may cause problems of specificity. These possibilities were tested using a series of one- and two-step assays.

#### **(i) One-step (a)-(a) assay.**

Microtitre plates were coated with a polyclonal anti-apo (a) antibody (Immuno) and 100µl of a series of Lp(a) standards (diluted 1:500) were pipetted, together with 100µl of europium-labelled anti-apo (a) detection antibody, into each well in a one-step procedure. This reaction mixture was incubated for three hours at 20°C and the assay completed using the standard protocol. Figure 5.10 shows the standard curve obtained using this procedure which reaches a plateau at high Lp(a) concentrations. Although increased sample dilution improved the linearity of the standard curve (data not shown), the theoretical problem of specificity resulting from use of the same antibody to both capture and detect Lp(a) precluded its further use.

#### **(ii) One- and two- step 35f5-(a) or 29c10-(a) assays.**

The data presented in this section was obtained using the 35f5-(a) assay. The 29c10-(a) assay was shown to perform in a similar way (data not shown). A comparison of one-, versus two-step assays using a final sample dilution of 1:4000 and labelled antibody diluted at 1:1000 was undertaken. The one-step assay was incubated for six hours while the two-step assay had two 3 hour incubations. All incubations were at 20°C. Figure 5.11 shows standard curves produced using each

assay format. It is apparent that both assays generate essentially linear standard curves with the one-step assay giving slightly higher fluorescence counts.

The performance of these assays at very high Lp(a) concentrations was investigated in an attempt to determine the concentration at which any 'high-dose hook effect' <sup>(234)</sup> might occur. The Lp(a) standard was diluted 1:10 to give a Lp(a) concentration well above the highest seen in human serum, and serially diluted to 1:1000. These dilutions were analysed using one- and two-step assays.

Figure 5.12 demonstrates that at very high analyte concentrations the one-step assay shows a 'hook' while the two-step assay does not. The arrow on the graph represents the Lp(a) concentration corresponding to a 1600 mg/L standard assayed using 200µl of a 1:4000 sample dilution. From this data it appears that no linearity problems would be encountered with either assay format at the Lp(a) concentrations seen in human serum (up to ~2000 mg/L). However, it was decided to abandon the one-step assay in favour of the two-step due to the better precision of the latter.

### **(iii) One- and two-step 35f5-29c10 assays.**

In spite of the low europium incorporation into the 29c10 antibody (0.23 Eu/antibody) (section 4.3.3), viable assays were developed. Microtitre plates were coated with 35f5 and one- and two-step assays were performed using a final sample dilution of 1:500 and a labelled antibody dilution of 1:1000. Two incubations each of 2 hours were used for the two-step assay and a single 4 hour incubation was used for the one-step procedure. The resulting standard curves (presented in figure 5.13) were very similar, therefore either assay was suitable for quantitation of Lp(a).

### **(iv) 29c10-29c10 two-step assay.**

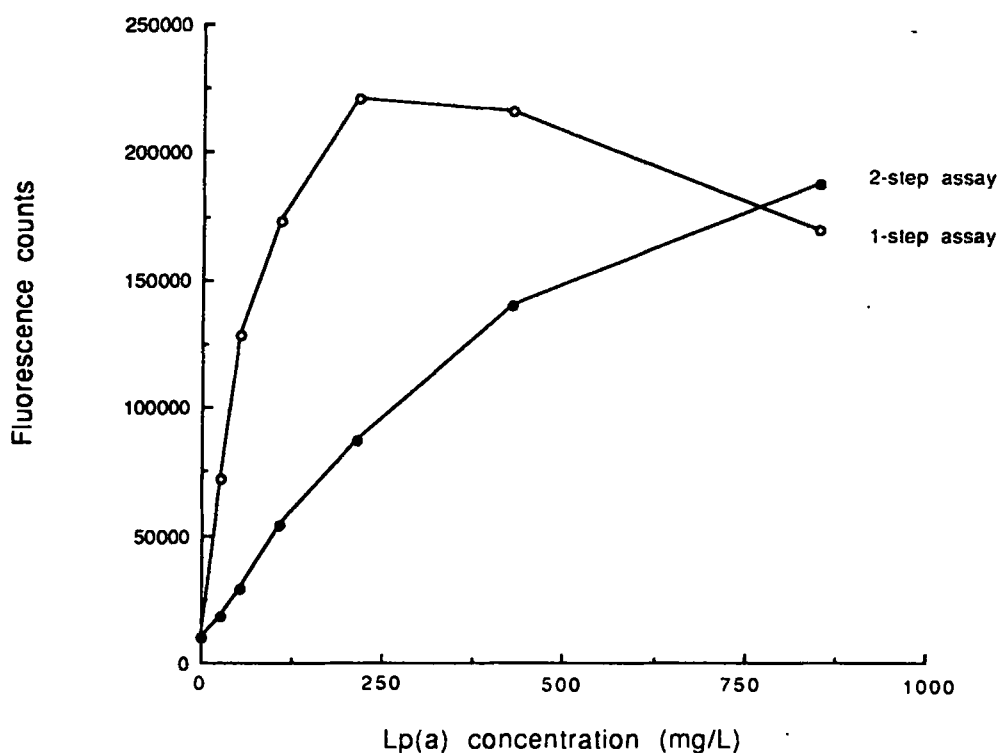
A two-step assay was designed using the 29c10 antibody to both capture and detect Lp(a). A series of Lp(a) standards (diluted 1:500) were assayed using microtitre plates coated with 29c10 and a 1:1000 dilution of europium-labelled 29c10.

However, although theory suggests that two identical monoclonal antibodies should not be able to bind at the same site on an antigen, this antibody combination did generate a standard curve for Lp(a) (figure 5.14) : indeed the fluorescence counts for the Lp(a) standards were similar to those produced using the 35f5-29c10 assay (figure 5.13). The data in figure 5.14 suggests that the 29c10 monoclonal antibody was able to bind to more than one site on the Lp(a) particle, probably to the kringle 4 domain (of which there are between 5-37 copies per Lp(a) particle). A detection antibody directed against this domain would be unsuitable for use in the Lp(a) assay since its binding to Lp(a) isoforms would be influenced by the number of kringle 4 repeat domains present in the isoform.

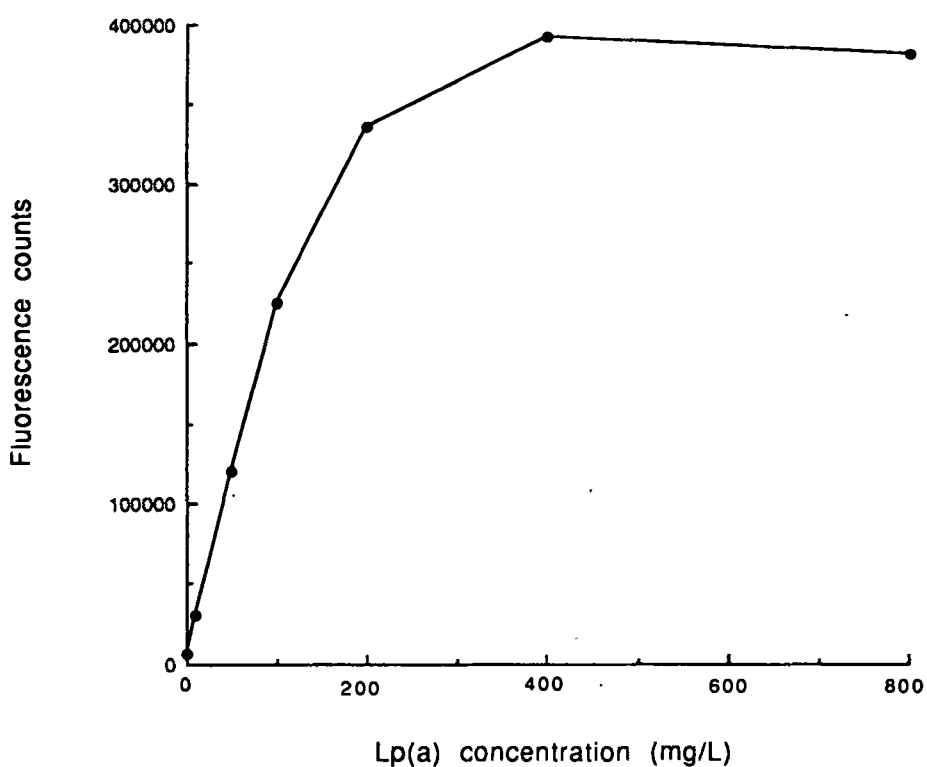
**(v) B-29c10 two-step assay.**

Although it was recognised that this antibody combination might present a problem of competition between apo B present in Lp(a) and apo B in other lipoproteins (for capture antibody binding sites), a two-step assay was, nevertheless, developed and a linear standard curve was obtained (data not shown).

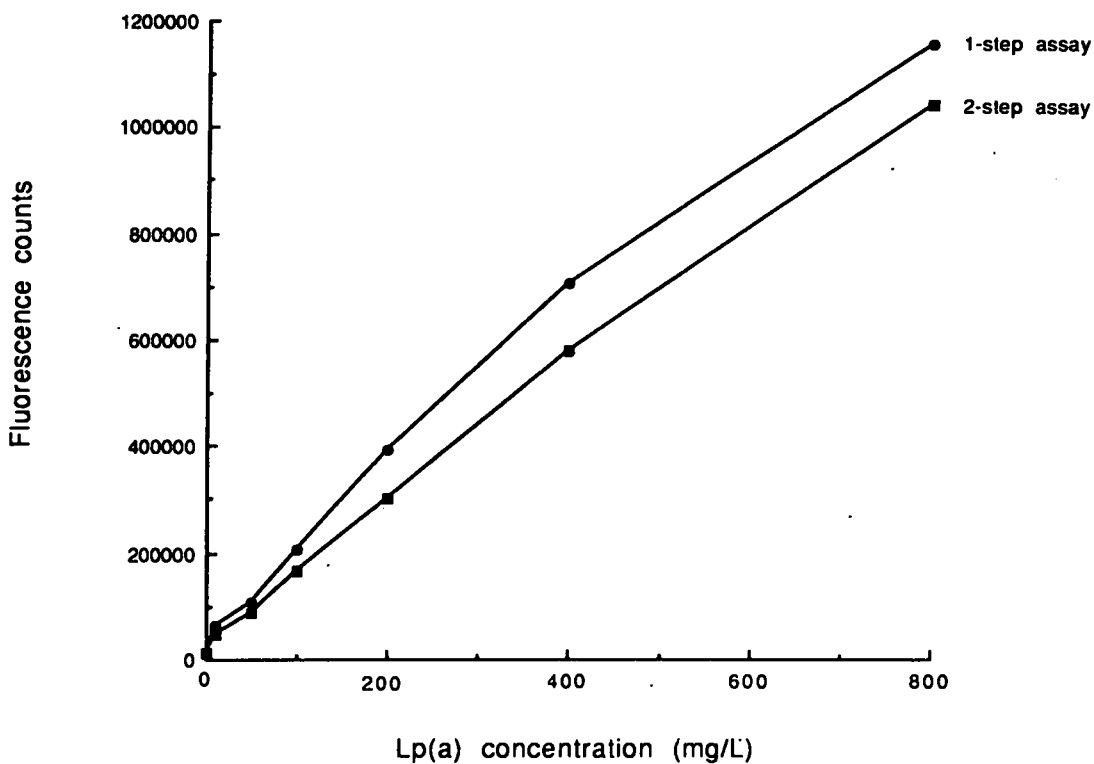
In summary, the choice of assay format was found to be highly dependant upon the antibody combination to be used. Assays employing two anti-apo (a) antibodies performed equally well in both the one- and two-step protocols. However, when using an anti-apo B detection antibody it was essential to employ a two-step format to avoid competition between apo B in Lp(a) and apo B present in other lipoproteins for binding to the detection antibody. Overall, each of the antibody combinations produced reasonably linear standard curve particularly when a two-step assay was used. A two-step assay using the 29c10-B antibody combination was selected to quantitate Lp(a) in patient samples since this combination was considered less likely to be influenced by size variations between the Lp(a) isoforms.



**Figure 5.9** Lp(a) assay using an anti-apo (a) capture antibody and anti-apo B detection antibody. Assay performed using one or two reagent addition steps.

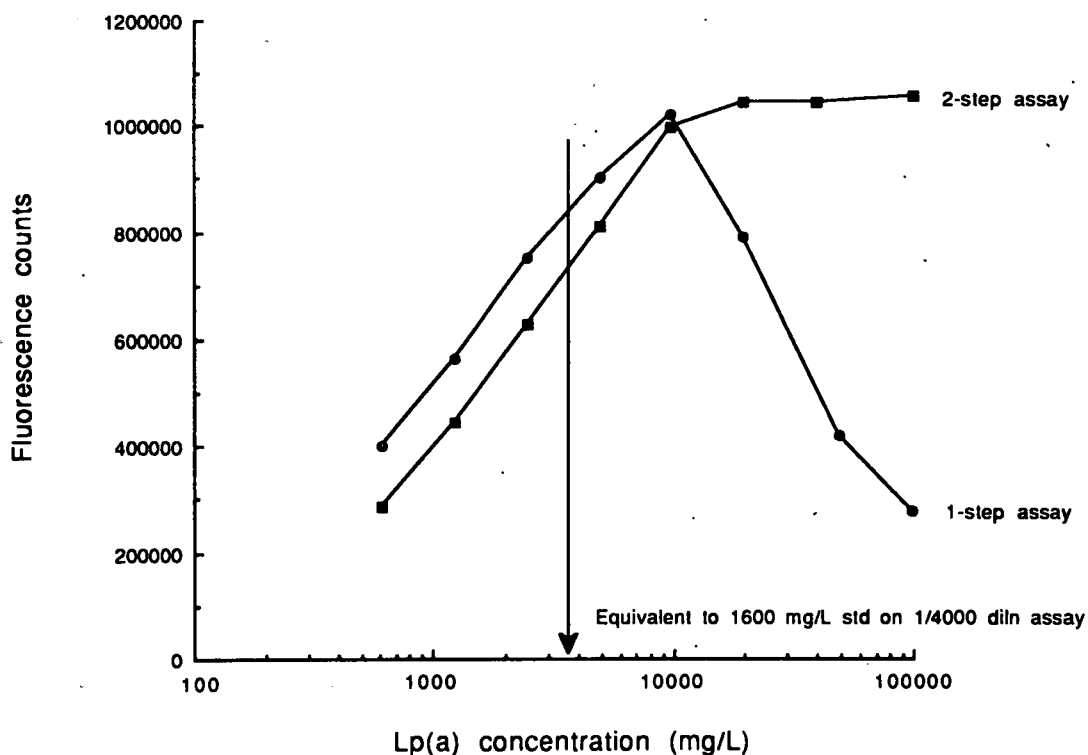


**Figure 5.10** Lp(a) standard curve from a 1-step assay using 'Immuno' anti-apo (a) antibody to both capture and detect Lp(a).



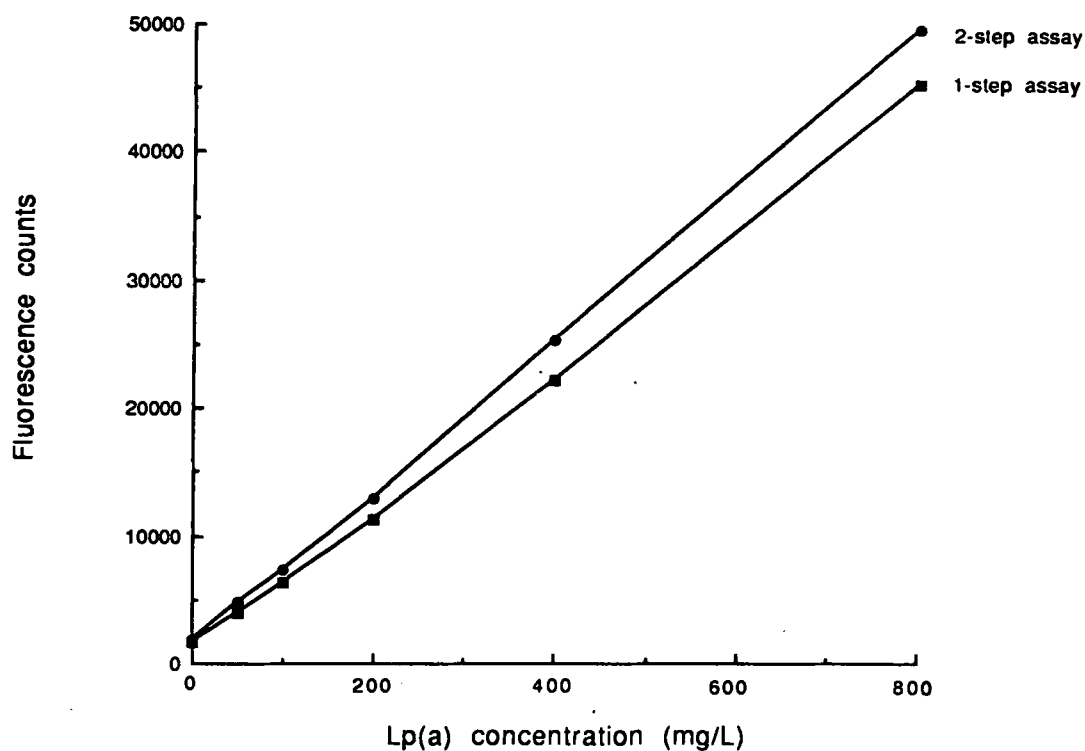
**Figure 5.11** Lp(a) standard curves obtained using the 35f5-(a) assay with one or two incubation steps.

35f5 capture antibody / Europium-labelled polyclonal anti-apo (a) detection antibody



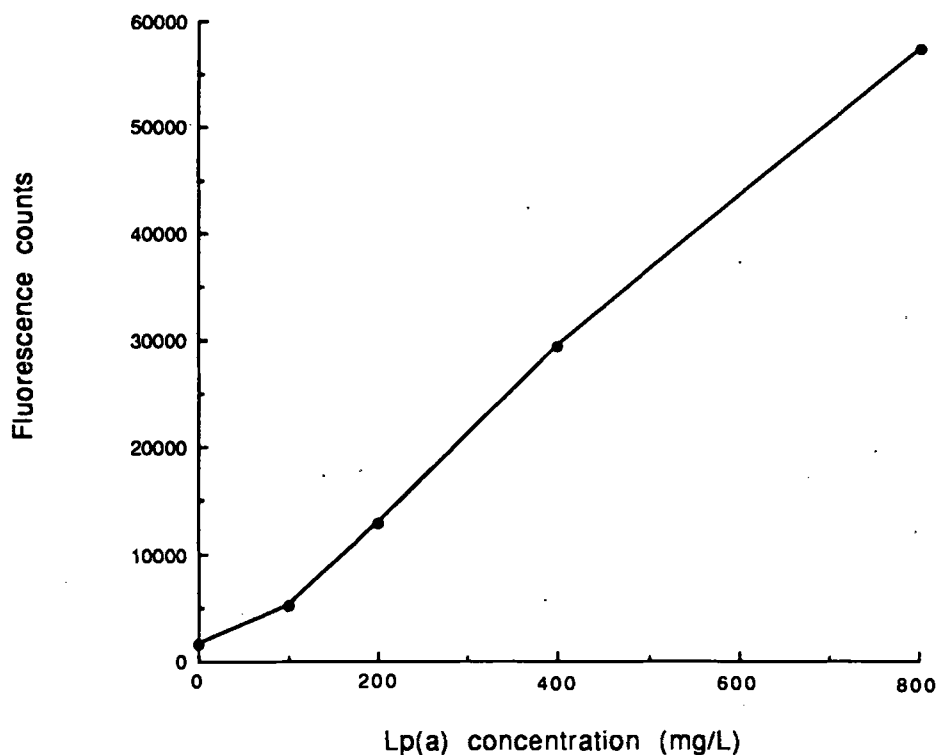
**Figure 5.12** A demonstration of the 'high dose hook' effect using the 1-step 35f5-(a) Lp(a) assay.

35f5 capture antibody / Europium-labelled polyclonal anti-apo (a) detection antibody



**Figure 5.13** Lp(a) standard curves from 1- and 2-step assays using the monoclonal antibodies 29c10 and 35f5.

35f5 capture antibody / Europium-labelled 29c10 anti-apo (a) detection antibody



**Figure 5.14** Standard curve from a two-step Lp(a) assay using 29c10 monoclonal antibody to both capture and detect Lp(a).

## **5.6 Sample dilution.**

It was apparent that serum samples would require dilution before analysis on the time-resolved fluorescence assay since commercial ELISA and IRMA kit assays use 1:42 to 1:500 predilution of samples prior to analysis. A 1:1000 sample dilution was chosen for the 29c10-B antibody combination as this produced a near-linear increase in fluorescence counts with increase in analyte concentration.

Because a large sample dilution was employed, it was possible to change the dilution factor of serum samples (without changing the dilution factor of the standard) in order to extend the sensitivity of assay further. For instance, a sample containing Lp(a) at less than 1 mg/L in an assay using a 1:1000 dilution may be reassayed at a 1:100 dilution and the result obtained divided by 10 to give a more accurate estimation of concentration.

## **5.7 Optimisation of europium-labelled antibody concentration.**

The labelled (detection) antibody in a two-site immunoassay is usually present in excess so that all of the molecules bound by the capture antibody are detected by the labelled antibody <sup>(234)</sup>. One important consideration when optimising the detection antibody concentration is that increasing additions of labelled antibody tend to increase non-specific binding (NSB). Low NSB is essential for a sensitive assay (see section 6.3.1).

### **Method**

In the following experiments, two-step assays were used. Lp(a) standard (800 mg/L at 1:1000 dilution) was incubated for 3 hours at 20°C using a 35f5 coated microtitre plate. After washing, 200µl of europium-labelled antibody (at various dilutions) were pipetted into each well and incubated for two hours at 20°C. The assay was then completed using the standard protocol.

### **5.7.1 Anti-apolipoprotein B detection antibody.**

A series of dilutions of the concentrated labelled antibody solution were made (1:1000 to 1:25) giving a range of concentrations from 31 to 1224 ng per well. Figure 5.15 plots fluorescence counts for the 800 mg/L Lp(a) standard versus amount of labelled antibody/well. It may be seen that a significant increase in fluorescence counts was achieved by increasing the amount of label added. The fluorescence counts for the zero standard increased from 800 for the 1:1000 labelled antibody dilution to 13500 for the 1:25 dilution. It appears that even at a 1:25 dilution an optimal labelled antibody concentration was not achieved. For subsequent assays the label was diluted to 1:1000 because at this concentration :-

- (i) A linear standard curve was obtained.
- (ii) Low and precise counts were obtained for the zero standard - thus improving assay sensitivity. Precision of fluorescence counts for the zero standard decreased as more label was added.
- (iii) There was a significant saving in labelled antibody : at a 1:1000 dilution the antibody from one labelling procedure would be sufficient for at least 170 microtitre plate assays whereas at 1:25 dilution only 4 assays could be run.

The reason that a true optimum label concentration was not established may be due to the nature of the Lp(a) particle. It is possible that more than one of the labelled polyclonal antibodies may bind to the relatively large apo B region of Lp(a). Therefore, by increasing the labelled antibody concentration, more labelled antibody may bind to apo B. Eventually saturation may be reached, although this appears to be at a very high labelled antibody concentration.

### **5.7.2 Anti-apolipoprotein (a) detection antibody.**

Figure 5.16 shows a series of Lp(a) standard curves obtained using the anti-apo (a) labelled antibody at dilutions of 1:1000 to 1:250 (23.5 to 94 ng/well). Fluorescence counts for the zero standard increased from 1800 to 5500 at these

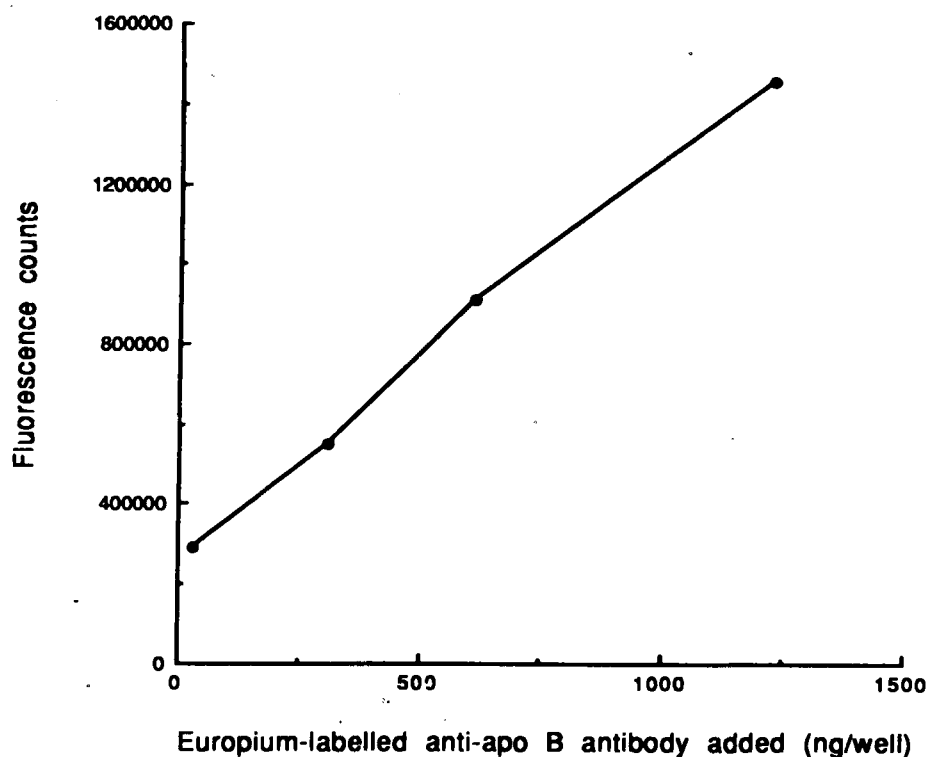


dilutions. For the reasons stated for the apo B detection antibody (section 5.7.1), the apo (a) detection antibody was used at 1:1000 dilution.

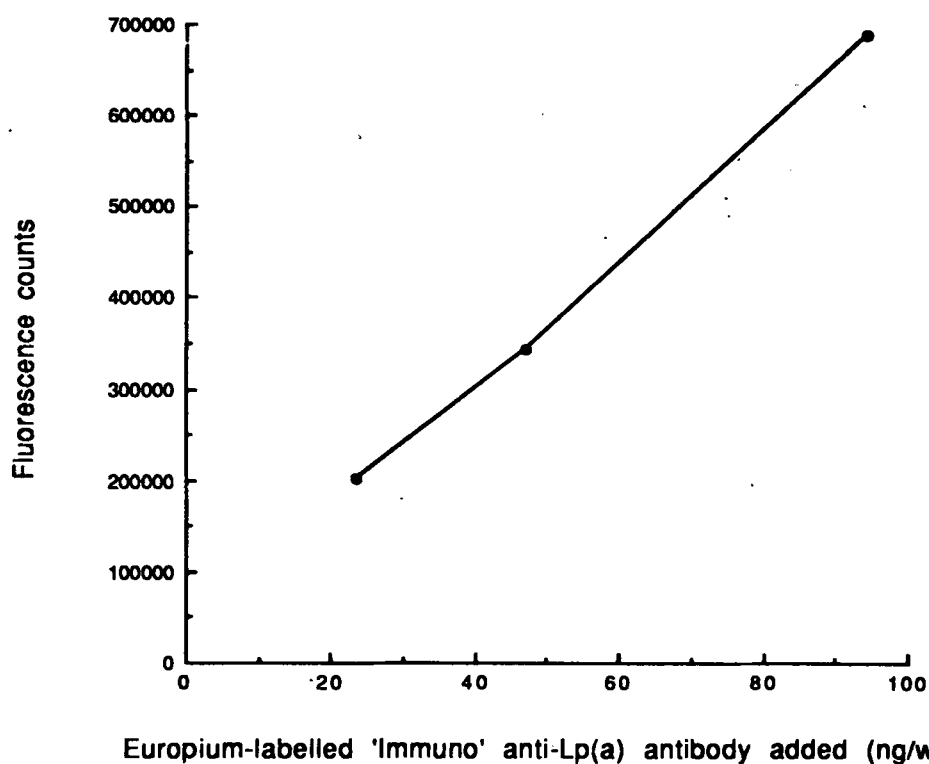
However, as with the anti-apo B antibody, an optimum dilution was not achieved. Like apo B, apo (a) is a large protein and it may therefore be possible for more than one labelled antibody to bind to it, especially if the antibody recognises epitopes on the kringle 4 domain which is present in multiple copies in Lp(a). It is likely that both of the europium labelled antibodies ('Immuno' anti-apo (a) and 29c10) bind to this domain, which makes them unsuitable for use as detection antibodies (as previously discussed - section 5.5).

### **5.8 Effect of microtitre plate agitation during incubations.**

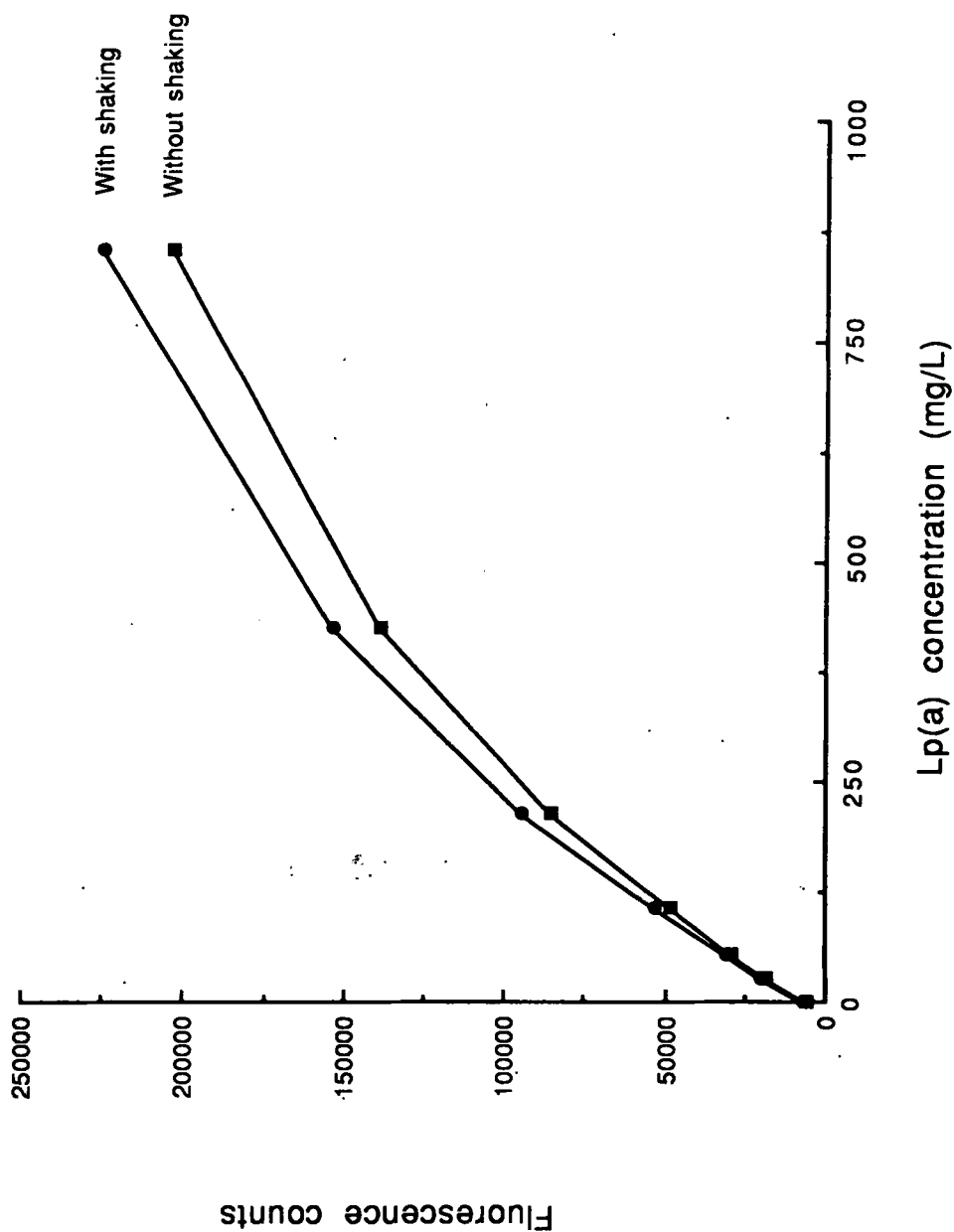
The effect of continuous shaking of the microtitre plate during incubations was investigated. Two sets of Lp(a) standards were analysed using 29c10-B assays; one plate was shaken continuously on a DELFIA® 1296-001 plateshaker, set at slow speed (approximately 80 vibrations/minute) and the other was left undisturbed. Figure 5.17 shows that a significant increase in fluorescence counts was achieved by continuously shaking microtitre plates during incubations presumably because the agitation increased the probability of antibody-antigen interactions. Continuous shaking of microtitre plates during incubations was used throughout assay development.



**Figure 5.15** Effect of increasing anti-apo B detection antibody concentration on fluorescence counts for an 800 mg/L Lp(a) standard.  
35f5 capture antibody



**Figure 5.16** Effects of increasing concentration of anti-apo (a) detection antibody on fluorescence counts for an 800 mg/L Lp(a) standard.  
35f5 capture antibody



**Figure 5.17** Lp(a) standards assayed with and without shaking of microtitre plates.

29c10 capture antibody / Europium-labelled polyclonal anti-apo B detection antibody

## **5.9 Optimisation of incubation times.**

One of the aims of this project was to develop an assay that could be completed within one (8-hour) working day. Therefore, since it was necessary to use a two-step assay, incubations had to be as short as possible.

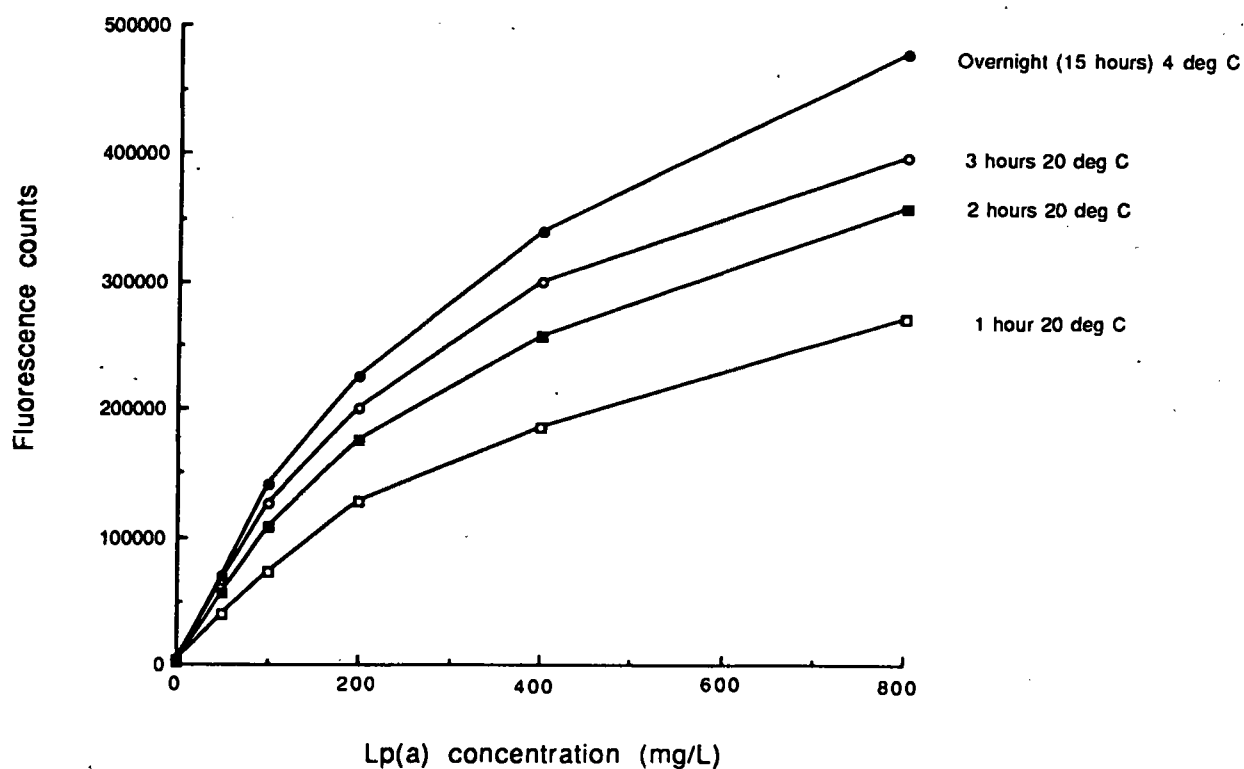
### **5.9.1 Sample incubation**

A series of Lp(a) standards (diluted 1:1000) were assayed on 35f5 coated microtitre plates using a two-step procedure. The diluted standards were incubated for 1-3 hours at 20°C or overnight at 4°C. The amount of standard bound to the plate was quantified using a 2 hour incubation at 20°C with the anti-apo B detection antibody. Data from the standard curves (figure 5.18) suggests that binding of Lp(a) to the 35f5 capture antibody was incomplete even at three hours.

### **5.9.2 Labelled antibody incubation.**

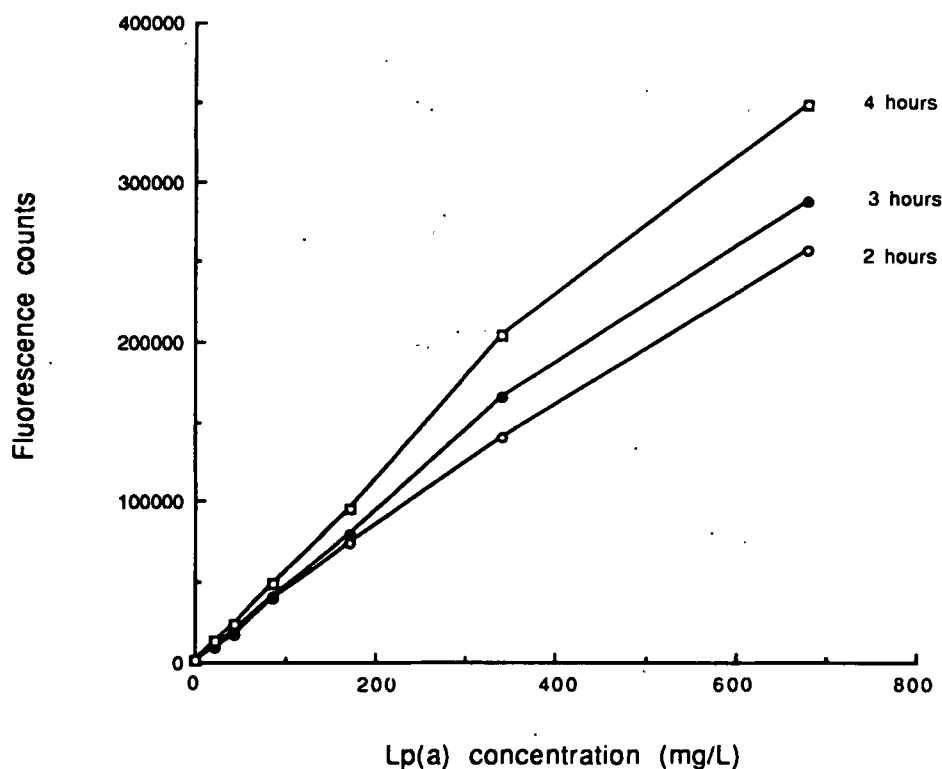
Three sets of diluted Lp(a) standards were assayed using 35f5 coated microtitre plates. Following washing, the assay was completed by addition of 200µl of europium labelled anti-apo B detection antibody to each well which was incubated for either 2, 3 or 4 hours at 20°C. The binding of this anti-apo B detection antibody to the captured Lp(a) was time-dependant (figure 5.19), maximum binding was not reached at 4 hours.

A compromise of a sample incubation time of 3 hours together with a 2 hour labelled antibody incubation time was subsequently employed. These sub-optimal incubation periods may possibly lead to problems of assay drift (gradual changes in results throughout an assay due to samples at the beginning and end a batch being incubated for slightly different times). This possibility was assessed by analysing serum samples both at the start and at the end of each batch. Comparison of results for these samples showed no consistent upwards or downwards trend, which suggests that systematic assay drift did not occur.



**Figure 5.18** Lp(a) standard curves from a 2-step 35f5-B assay. Using sample incubation times from 1-15 hours.

35f5 capture antibody / Europium-labelled polyclonal anti-apo B detection antibody



**Figure 5.19** The effect of increasing incubation times with europium labelled anti-apo B antibody on Lp(a) standard curves.

35f5 capture antibody / Europium-labelled polyclonal anti-apo B detection antibody

## **5.10 Standardisation and quality control.**

Since Lp(a) assays are seldom performed in the United Kingdom, a wide selection of different commercial calibrants and quality control materials are not readily available. The following materials were used as calibrants and quality controls in the time-resolved fluoroimmunoassay of Lp(a).

### **5.10.1 Standardisation.**

A commercial preparation from 'Immuno' was used in this project (see materials and methods section). Analysis of three different batches of standards (each with an assigned Lp(a) concentration) in one 29c10-B assay showed between-batch variability of up to 20% (data not shown). The manufacturers quote that the standard was prepared from pooled human plasma and calibrated by comparison with purified Lp(a) using an electroimmunodiffusion technique <sup>(235)</sup>. Since the standard was said to contain a mixture of S2 and S3 isoforms, the between-batch variability observed may be due to each standard preparation containing different proportions of these isoforms. This inter-standard variability has serious implications when long term studies employing Lp(a) assays are performed using more than one batch of standard.

The Lp(a) standards had concentrations between 680-850 mg/L. Since a small proportion of patients have serum Lp(a) concentrations above 850 mg/L they would require reanalysis after predilution. To extend the working range of the assay, the standard was therefore diluted half as much as the unknowns (i.e. 1:500 in an assay where the unknowns were diluted 1:1000) to effectively double the standard concentration.

### **5.10.2 Quality control.**

No specific quality control materials are available commercially. A series of clinical chemistry control materials were therefore analysed in the hope of identifying some with suitable concentrations of Lp(a). However, many were animal serum-based preparations which contain no Lp(a), and those human serum preparations that

did contain Lp(a) had concentrations below 200 mg/L. Ideally, for Lp(a) assays a series of controls with concentrations of approximately 50, 300 and 600 mg/L would be required. In order to obtain controls with these Lp(a) concentrations, serum was obtained from the National Blood Transfusion Service (Cambridge) which had been screened for Hepatitis A and B and HIV virus. After measurement of Lp(a), suitable samples were divided into 30µl aliquots, frozen at -20°C, and analysed in each Lp(a) assay to ensure the quality of the results.

### 5.11 Assay costings.

An important parameter in the assessment of an assay kit or newly developed method in today's routine Clinical Chemistry laboratory is cost. The following assay costings are compared to one of the more commonly used ELISA immunoassays, the Immuno Immunozyt<sup>®</sup> Lp(a) kit which costs £215.00. This kit has sufficient materials to analyse up to a maximum of 36 patient samples in duplicate, making a reagent cost of £5.97 per sample.

The cost of the time resolved fluorescence assay per assay (with samples analysed in duplicate) may be estimated as follows :-

Microtitre plate	£ 0.50
BSA for plate blocking. (£284.60/100 g) 0.25g used/plate	£ 0.71
Lp(a) standard (£23.00 for 0.5 ml) Sufficient for 15 assays therefore cost/assay	£ 1.53
Binding site anti-apo B antibody (£35/ml) 0.6 ml may be used for each labelling.	£21.00/labelling
Europium Labelling kit	£146 (with 20% discount )
Therefore antibody + labelling kit costs £167.00 This should produce sufficient labelled antibody for 150 assays at least. Therefore cost per assay	<u>£ 1.11</u> total £ 3.85

Other consumables :-Sample tubes, pipette tips,  
Buffers (assay, wash, enhancement solution supplied in excess with commercial kits),

Chromatography materials for labelled antibody preparation,  
Preparation of the monoclonal antibodies (difficult to cost).

The total cost of these per assay should not exceed £5.00.

Therefore 36 samples may be analysed in duplicate, at a cost of no more than £8.85, giving a cost of £0.25 per patient sample.

As the staff time required to perform both assays is similar this represents a considerable saving on the commercial kit assays

## **5.12 DISCUSSION.**

The findings presented in this chapter emphasise the need for careful optimisation of immunoassays. Time-resolved fluorescence immunoassays for Lp(a) present some particular analytical problems, in that the antibodies used dictate, to some extent, the assay design. In retrospect, a monoclonal anti-apo (a) capture antibody (directed against an epitope other than the kringle 4 domain) together with a monoclonal anti-apo B detection antibody might be the most suitable antibody combination.

It was hoped that the use of a monoclonal anti-apo(a) capture antibody directed against the kringle 4 domain of Lp(a) would not have different affinities for the various Lp(a) isoforms. This may only be verified by analysing at a series of samples with known isoforms using various anti-apo (a) capture antibodies. A satisfactory electrophoretic method for isoform determination has not yet been completed by the author in-house, although several immunoblotting methods have recently been described (38,236).

Commercial ELISA assay kits use assay protocols similar to the those for the time-resolved fluorescence assay described here. Thus, assays using an anti-apo (a) antibody to both capture and detect Lp(a) use a one-step incubation, while those using an anti-apo (a) capture antibody with an anti-apo B detection antibody use a two-step procedure. Sample dilutions vary from kit to kit (1:42 - 1:500) but are in the same region as the time-resolved fluorescence assays. Since the commercial assay kits are supplied with the microtitre plate already coated with antibody and blocked with



BSA, details of these procedures are not available. Incubation times for the ELISA assays are shorter than the time-resolved fluorescence assay, possibly because both sample and labelled antibody incubation times have not been optimised. Personal experience with the Immunozy<sup>®</sup> ELISA assay indicates that 'drift' (section 5.9.2) occurs across the microtitre plate, this may be due to the short incubation times or to temperature variations across the plate which affect enzyme activity. The time-resolved fluorescence assay is performed at room temperature and, because it does not employ any enzymes, is less sensitive to temperature variations.

## **Appendix 5.1**

**Assay protocol for a time-resolved fluorescence Lp(a) assay using a monoclonal anti-apolipoprotein (a) capture antibody (29c10) and a europium labelled polyclonal anti-apolipoprotein B detection antibody.**

### **Coating of microtitre plates with antibody.**

Dilute 29c10 antibody 1:1000 in bicarbonate buffer pH 9.2, mix well. Pipette 250µl of solution into each microtitre plate well. Cover and incubate at 4°C overnight (or 1 hour at 20°C)

Empty the wells (save the antibody solution) and wash the plate 3 times with wash buffer using the DELFIA® automated plate washer (400µl wash buffer/wash).

Pipette 300µl of bovine serum albumin solution (10g/L in phosphate buffered saline) into each well, incubate at 20°C for 1 hour.

Wash microtitre plate once on the automated plate washer (400µl wash buffer/wash). The plate is now ready for use. It may be stored at 4°C in a closed, desiccated container for up to three months if required.

### **Sample preparation/application.**

Dilute Lp(a) standard (current lot number 110/170 (680 mg/L)) in DELFIA® assay buffer. A 1:500 dilution of the standard is equivalent to 1360 mg/L in an assay where samples and quality control materials are diluted 1:1000. Serially dilute the 1360 mg/L standard (1:2) in assay buffer to give a series of standards with Lp(a) concentrations of 680, 340, 170, 85, 42.5, 21.2, 10.6 mg/L. Assay buffer is used as a zero standard.

Thaw, mix and centrifuge patient samples and quality control materials, dilute 1:1000 in DELFIA® assay buffer.

Pipette 200µl of diluted standards, quality controls and patient samples in duplicate to the microtitre plate. Quality control materials should be included both before the first and after the last patient sample.

Incubate the microtitre plate for three hours at 20°C (with Slow shaking on the DELFIA® plate shaker).

Wash the plate four times on the automated plate washer (400µl wash buffer/wash).

Add 200µl of europium-labelled anti-apolipoprotein B antibody (diluted 1:1000 in DELFIA® assay buffer) to each well.

Incubate the plate for two hours at 20°C (with slow shaking on the DELFIA® plate shaker).

Wash the plate six times on the automated plate washer (400µl wash buffer/wash).

Add 200µl of DELFIA® Enhancement solution to each well.

Incubate the plate for ten minutes at 20°C with shaking on the DELFIA® plate shaker.

Allow to stand for 5 minutes before reading.

Read the plate on the DELFIA® research fluorimeter.

Use Pharmacia Multicalc software to produce a standard curve and to calculate unknowns from the fluorescence counts.

**CHAPTER 6**  
**PERFORMANCE CHARACTERISTICS**  
**OF THE TIME-RESOLVED FLUORESCENCE  $L_p(a)$  ASSAY**

## 6.1 INTRODUCTION.

Careful optimisation of each incubation step in an immunoassay (as described in chapter 5) should produce an assay with acceptable analytical performance. The assay should also be robust so that it may be performed by many different operators without loss of performance, and stable so that satisfactory analytical performance may be sustained over many years.

One aim of this project was to produce a simple, reliable and cheap assay that outperformed any commercial kit currently available in the United Kingdom with regard to sensitivity, assay range and reproducibility. It was hoped that this improved sensitivity would allow quantitative measurement of serum Lp(a) in all human subjects, where, in some previous studies none was detected<sup>(33)</sup>.

At the outset of this project targets were set for analytical performance of the assay, it was hoped that sensitivity below 5 mg/L with an assay working range up to 1000 mg/L might be achieved. This would ensure that Lp(a) could be measured in all serum samples without any additional pre-dilution steps. The assay should be precise enough to produce within-batch precision less than 6% and between-batch precision less than 10% across the working range of the assay<sup>(237)</sup>. The assay should be easy to perform within a working day, it should require a small sample volume and it should be free from interferences from other biomolecules present in human serum (both in health and disease). The results obtained using the assay should not be grossly different from those obtained using other commercially-available assay kits.

## 6.2 PRECISION.

*Within-batch precision* was estimated by analysing patient serum samples with high, medium and low concentrations of Lp(a) (~600, 300 & 100 mg/L). Each sample was diluted according to the assay protocol and analysed, in duplicate, up to 12 times on one microtitre plate. The average value of the duplicate was then used to calculate the mean and standard deviation (SD) for each set of samples.

These figures were then used to calculate the coefficient of variation (CV) of the results using the equation (238):- 
$$CV = \frac{SD}{\text{mean}} \times 100. \quad (\%)$$

*Between-batch precision* was assessed using three serum samples with high, medium and low concentrations of Lp(a) (as above). Samples were divided into aliquots and frozen at -20°C. The samples were analysed in 20 separate assays. A fresh aliquot of each sample was thawed, mixed and centrifuged for each assay. The samples were analysed, in duplicate, and the mean value was recorded. The mean and SD of these concentrations were then used to calculate between-batch CV as above. These samples were used as internal quality control materials for the Lp(a) assay as commercial quality control preparations with suitable levels of Lp(a) are not readily available.

*A precision profile* was used to assess precision across the working range of the assay. The precision profile was calculated using data from Lp(a) assays and therefore requires no extra analytical work. This 'standard deviation of duplicates' (SD dups) computer program, written by Dr P. Raggatt (Dept Clinical Biochemistry, Addenbrooke's Hospital, Cambridge) requires analyte concentrations to be assigned to one of seven 'bins', which should span the assay working range. The individual Lp(a) concentrations from each pair of replicates is entered into the program which then calculates the mean of each duplicate, the SD and percentage difference of the replicates. For reliable analysis at least fifty values should be present in each of the bins. Once sufficient data is entered, the program calculates the mean of the duplicates in each bin. The mean SD of the duplicates of each result and the CV is calculated from these results. A plot of bin mean Lp(a) concentration against CV gives an estimate of assay precision across the analyte range. An assay with coefficients of variation below 8% across the assay range may be regarded as precise (237).

## 6.2.1 Results.

### Within-batch precision.

Table 6.1 shows within-batch precision data for various combinations of capture and detection antibodies at three analyte concentrations.

**Table 6.1**

Antibody combination	n	mean [Lp(a)] (mg/L)	SD (mg/L)	CV (%)
(a)-(a)	12	71	3.5	5.0
	-	-	-	-
	12	717	15.5	2.2
(a)-B	10	41	1.2	2.8
	10	354	18.1	5.1
	10	527	38.6	7.3
29c10-B	10	96	5.8	6.0
	10	311	11.2	3.6
	10	559	12.9	2.3
35f5-B	10	108	2.8	2.6
	10	304	13.9	4.6
	10	441	17.6	4.0
35f5-(a)	12	40	1.9	4.7
	12	397	10.3	2.6
	12	825	33.0	4.0
35f5-29c10	11	25	1.8	7.2
	11	398	5.4	1.4
	11	855	3.9	4.6

Abbreviations for the antibodies used :

(a) = 'Immuno' polyclonal anti-apo (a) antibody;

B = 'Binding Site' polyclonal anti-apo B antibody;

29c10 or 35f5 in-house monoclonal anti-apo (a) antibodies.

### **Between-batch precision.**

For reliable between-batch precision studies, it is accepted practice that data from at least 20 separate assays is required.

The only antibody combination in which 20 Lp(a) assays were run was 29c10-B.

Data obtained at 4 analyte concentrations from the 20 assays is shown in table 6.2

**Table 6.2**

Between batch precision data for the 29c10-B time-resolved fluorescence Lp(a) assay at 4 analyte concentrations.

Mean (mg/L)	SD (mg/L)	CV (%)
39.5	3.4	8.6
157	10.8	6.9
407	26.6	6.5
940	70.5	7.5

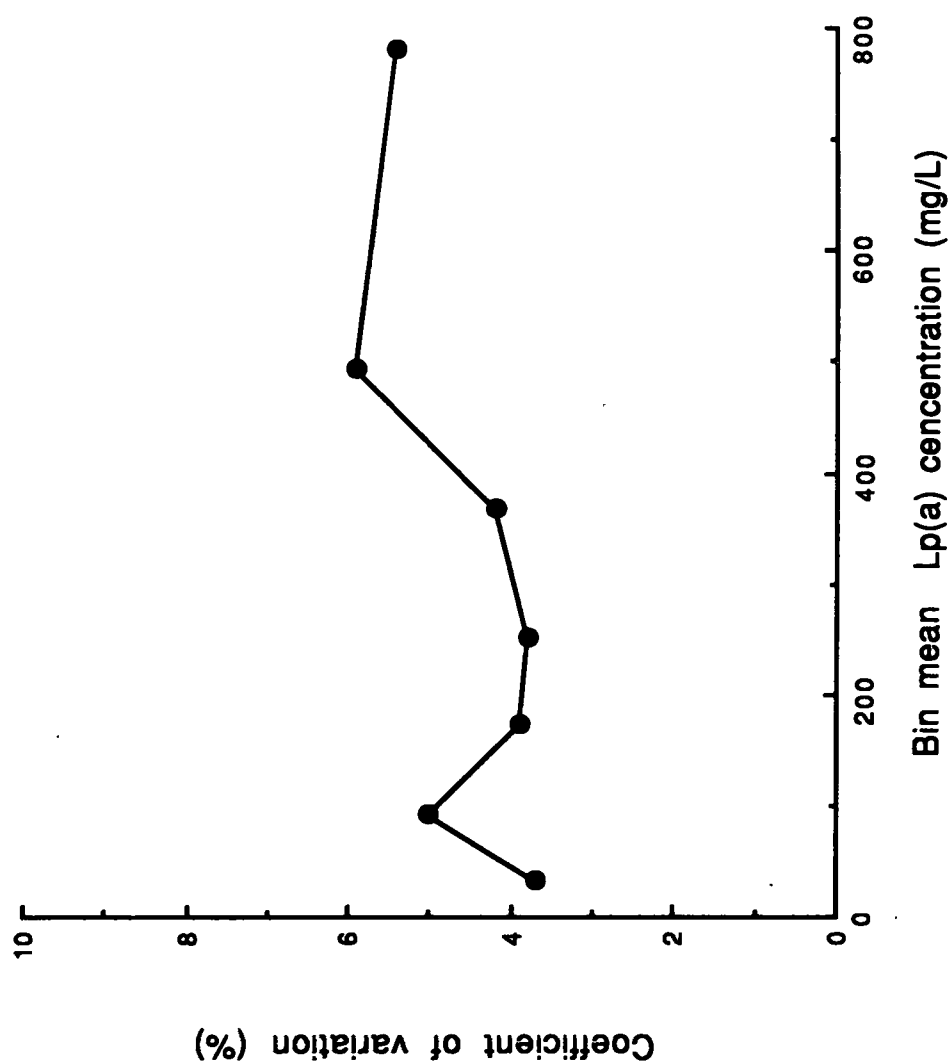
Limited data (from 8 assays) suggested a similar between-batch precision could be achieved using the (a)-B assay (data not shown).

### **Precision profile.**

This was assessed only for the 29c10-B assay. At least 25 data points were present in each bin. In total 203 pairs of results were used in the calculations, 172 (85%) of them had less than 10% difference between the replicates (which is very good for an immunoassay), a further 16 (8%) had differences between 10 and 15 % and the remaining 13 (7%) had differences greater than 15%.

A plot of bin mean against %CV is shown in figure 6.1.





**Figure 6.1** A precision profile for the 29c10-B time-resolved fluorescence Lp(a) assay. Profile calculated using the 'SD Duplicates' computer program.

### **6.2.2 Discussion.**

The precision data obtained in the time-resolved fluorescence assay for each antibody combination is well within limits deemed to be acceptable for an immunoassay <sup>(237)</sup>. The data compares favourably with that produced by manufacturers of commercial ELISA assays who quote between-batch CV in the region of 5-10 % and within-batch CV in the order of 3-8 % <sup>(214-218)</sup>. In practice, the Immunozy<sup>®</sup> ELISA assay used in this department has a between-batch variation in excess of 10% for quality control materials with Lp(a) concentrations of 250 and 350 mg/L. Improvements in assay precision may be made in two ways. Firstly, use of a robotic sample pipetting station such as the Tecan RSP 3032 would almost certainly improve precision of duplicates when compared to manual pipetting techniques. Secondly, the preparation of standard materials may be improved; the current practice of diluting and double diluting an aliquot of standard for each assay is prone to errors and between-batch variation. Improvements may be made by diluting the stock standard material to produce a series of standards which are then frozen in aliquots. These aliquots would then be diluted in assay buffer (i.e. 1:1000 for the 29c10-B assay).

## **6.3 SENSITIVITY.**

### **6.3.1 Method.**

The sensitivity of an immunoassay may be defined as the lowest analyte concentration that may reliably be distinguished from zero. This is usually estimated by analysing at least twenty replicates of the zero standard (assay buffer), together with a standard curve, in one assay. The mean and standard deviation of the zero counts are calculated and the analytical sensitivity is determined as the analyte concentration at which the (mean + 3 x SD) counts intercept the standard curve <sup>(231)</sup>.

### 6.3.2 Results.

Mean (+ SD) of fluorescence counts from 20 replicates of the zero standard are presented in table 6.3, together with counts for the lowest standard the assay. This data was used to assess sensitivity as described in 6.3.1, in cases where the (mean + 3 SD) counts were less than the lower standard, the response between zero and that standard was assumed to be linear.

**Table 6.3**

Mean (+ SD) of fluorescence counts from 20 replicates of the zero standard in the 29c10-B time-resolved fluorescence Lp(a) assay. The data was used to estimate assay sensitivity.

Antibody Combination	Zero std counts Mean	SD	Std counts (3 mg/L)	Sensitivity (mg/L)
35f5-(a)	1463	104	2425	1
35f5-29c10	1087	72	1602	2
29c10-B	865	62	1451	1

Data from the 35f5/B and (a)/B assay (not shown) also produced assay sensitivities of 2 mg/L.

A sensitivity of 1 mg/L in an assay using 200 $\mu$ l of a 1:1000 sample dilution means that, in absolute terms, 1 $\mu$ g/L of lipoprotein (a) is detected.

The sensitivities achieved for the time-resolved fluorescence Lp(a) assays are superior to those quoted for commercial kits. ELISA kit manufacturers claim sensitivities of between 10 and 20 mg/L <sup>(214,216)</sup>. Information from Immuno suggests that their immunoturbidimetric assay has a sensitivity of 50 mg/L and the Pharmacia IRMA assay has a sensitivity of around 3 mg/L <sup>(218)</sup>.

To some extent assay sensitivity is dependant upon the antibody combination employed. For instance, if the detection antibody was directed against the kringle 4

repeating domains of Lp(a) then, even at low analyte concentrations, it may be possible to bind several labelled antibodies, thus giving a relatively large fluorescence signal. Conversely, if an anti-apo B detection antibody was used, it may only be possible to bind only one detection antibody, therefore giving a lower fluorescence signal and lower sensitivity.

The 1:1000 sample dilution employed in the 29c10-B assay means that if greater sensitivity is required, a lower sample dilution may be used, (e.g. 1:100) which should improve the apparent sensitivity to 0.2 mg/L. Whether this degree of sensitivity would ever be necessary when measuring Lp(a) in a human serum sample is debatable. However, it may be useful (as a research tool) in detecting the presence of Lp(a) in other body fluids or cell culture supernatants.

## **6.4 LINEARITY ON DILUTION.**

### **6.4.1 Method.**

Measured [analyte] in samples analysed by immunoassay should decrease in proportion to sample dilution. However, at lower analyte concentrations other factors (such as assay imprecision) may produce non-linear dilution. Failure of samples to dilute in a linear fashion may also be due interfering factor(s) which only influence the assay at high concentration (i.e. the interfering factor may be 'diluted out'). To assess linearity, a series of at least two dilutions were made on a series of samples with elevated Lp(a) concentrations. The undiluted sample plus 1:2 & 1:4 dilutions (in phosphate-buffered saline, pH 7.4) were then analysed in duplicate in one batch and the measured Lp(a) concentration was plotted against dilution factor.

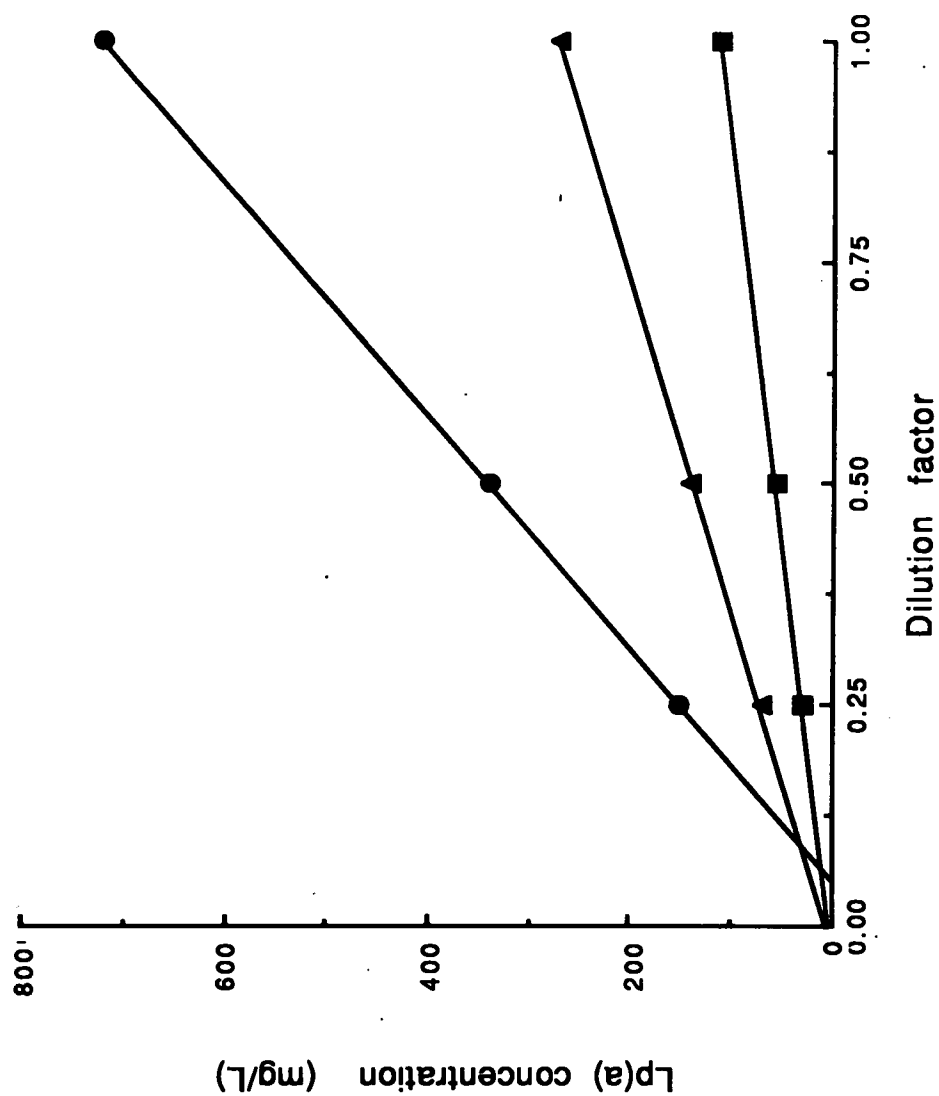
### **6.4.2 Results.**

Figure 6.2 shows a series of samples (with high and low Lp(a) concentrations) that were diluted and assayed using the 29c10-B antibody combination. The dilution factor refers to the proportion of the undiluted sample remaining (where the undiluted

sample has a dilution factor of 1.0). Samples diluted in assay buffer to the standard assay dilution of 1:1000 were then diluted further and assayed.

Dilution data presented graphically reveals linearity if the line of best fit between the points passes through the origin and the correlation coefficient of the fit between the points on the line ( $r$ ) is greater than 0.9. It is apparent from figure 6.2 that these criteria are achieved for these samples.

Similar dilution patterns were observed for most of the other antibody combinations (data not shown). However, dilutions in the (a)-(a) assay deviated somewhat from linearity, possibly because the same antibody was used to both capture and to detect the Lp(a). In practice, because of the wide working range of the time-resolved fluorescence assays, pre-dilution of patient samples should not be necessary. Other commercial assays with upper limits of measurement at around 700-800 mg/L require that some samples be pre-diluted.



**FIGURE 6.2** Dilution of three serum samples in the 29c10-B assay.  
A dilution factor of 0.5 represents a 1:2 dilution.

## **6.5 COMPARISON WITH OTHER Lp(a) ASSAYS.**

These comparisons may either be performed directly (i.e. analysis of a group of samples in each assay) or by comparison of data obtained from external quality control schemes.

### **6.5.1 External quality control schemes.**

During this project two external quality control schemes for Lp(a) were operating in this country (organised from Manchester and Bristol). Aliquots of 3-6 different serum samples were sent to each laboratory participating in the scheme at two-monthly intervals. The samples were analysed and the results were returned to the scheme co-ordinator together with details of the analytical method employed. Results are compared with those obtained by other participants using the same or differing methodologies. The aim of these schemes is to produce consistency of results amongst its users. For external quality control data to be valid, it is important that there is a sufficient number of participants in the scheme (ideally, at least fifty) to produce accurate mean values. For the Lp(a) schemes presented here, the maximum number of participants at any one time was fifteen, who used five different analytical methods.

53 samples from the Manchester scheme were analysed retrospectively after storage for up to two years at -70°C. Samples from the Bristol scheme were divided into aliquots and frozen at -20°C on receipt and analysed within seven days. All samples were thawed, mixed thoroughly and centrifuged before analysis using the 29c10-B assay. Pooled internal quality control sera were analysed together with these samples. The measured Lp(a) concentrations for these samples were within acceptable analytical ranges.

### **Results.**

The Lp(a) results obtained using the time-resolved fluorescence assay were plotted against the mean result (for that sample) from other laboratories using each

kit. These plots are not presented but correlation data from the 'line of best fit' through the data points is shown in table 6.4.

The correlation refers to the equation  $y = mx + c$  <sup>(238)</sup> where:-

$y$  = Time-resolved fluorescence assay result.

$m$  = Slope of the correlation line ;  $x$  = commercial assay result.

$c$  = intercept of the correlation line.

also :  $n$  = number of results in the correlation

$r$  = Correlation coefficient.

**Table 6.4**

Correlation data obtained from comparisons of Lp(a) results generated using an in-house time-resolved fluoroimmunoassay and 5 different commercially available diagnostic kits.

ASSAY	n	x	c	r
Biopool ELISA	50	0.76	-0.4	0.835
Innotest ELISA	25	1.00	-1.3	0.827
Immuno ELISA	50	0.966	-1.4	0.850
Pharmacia IRMA	50	1.22	2.5	0.878
Turbidimetry	50	0.88	6.0	0.876

This data demonstrates that, in general, there is a reasonably good correlation between the results produced by different assays ( $r > 0.8$ ). However, the slopes of the correlation lines (0.76-1.22) indicate that there is a difference in the measured Lp(a) concentrations. It was also apparent from the correlation plots (data not shown) that up to 2-fold variations in serum Lp(a) concentrations may occur when samples are analysed using different analytical methods.

A comparison of Lp(a) results for each sample obtained using the time-resolved fluorescence assay with the overall mean of results for the same sample measured using the other assays is shown in figure 6.3. The data indicates that a good correlation was obtained; the slope of the correlation line suggests that the time-



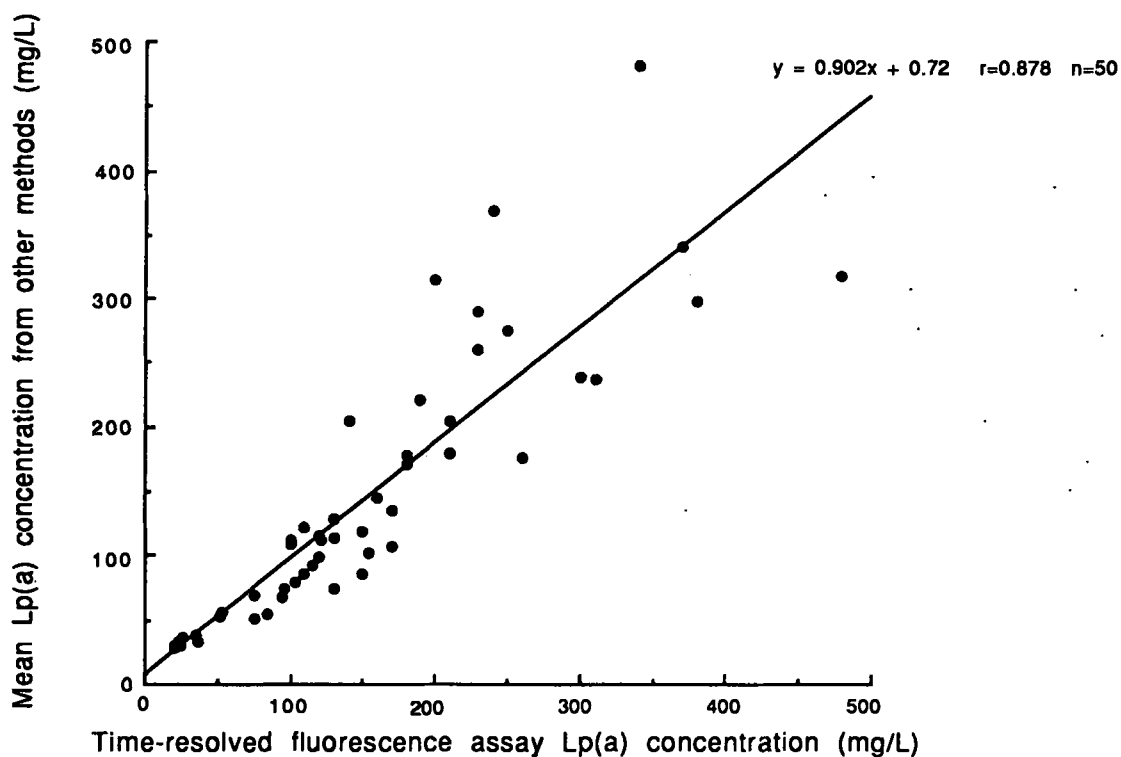
resolved fluorescence assay gives results ~10% higher than the mean result from other methods. The time-resolved fluorescence assay results were also expressed in terms of standard deviations from the mean result of all methods (figure 6.4). This plot showed 50/53 results fell within  $\pm 2 \times \text{SD}$  of the overall mean result for each sample. The  $\pm 2\text{SD}$  of the mean is widely accepted as representing acceptable analytical performance in an external quality control scheme <sup>(239)</sup>. However, in this scheme, (because of the small number of participants and wide analytical variation) the standard deviation for results obtained for each sample was quite large.

It was apparent that analysis of the external quality control samples would yield little useful information on the performance of the time-resolved fluorescence assay for the following reasons :

- (i) The most results returned for any one sample was 15 and for some samples there were only 6 results. A reliable estimate of mean and SD ideally requires at least 20 data points.
- (ii) Five different assays were employed, each using different antibody combinations and standards which is likely to exaggerate any between-laboratory variability.

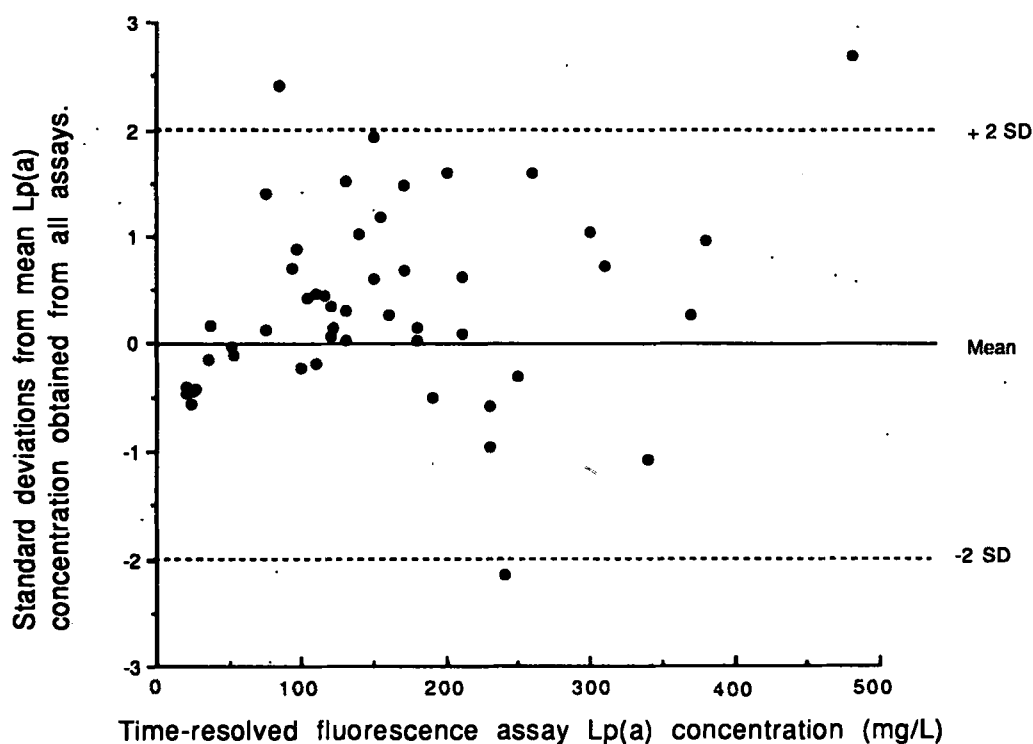
The only conclusions that can reliably be drawn from this study is that the time-resolved fluorescence assay is not drastically out of consensus with the others. However, since samples with Lp(a) concentrations above 500 mg/L have not been distributed in this scheme, it is possible that limitations of the analytical performance of commercial assays may be even more evident at this high concentration.

The Bristol QC scheme, as yet, has only 6 participants. This makes interpretation of results even less reliable than for the Manchester scheme. The results returned so far (9 samples) have again shown wide inter-laboratory variations (e.g. 360-890 mg/L on one sample). The time-resolved fluorescence assay gives results slightly greater than the overall mean.



**Figure 6.3**

Comparison of Lp(a) concentrations measured in external quality control samples. Result for the time-resolved fluorescence assay vs mean result from all other assays.



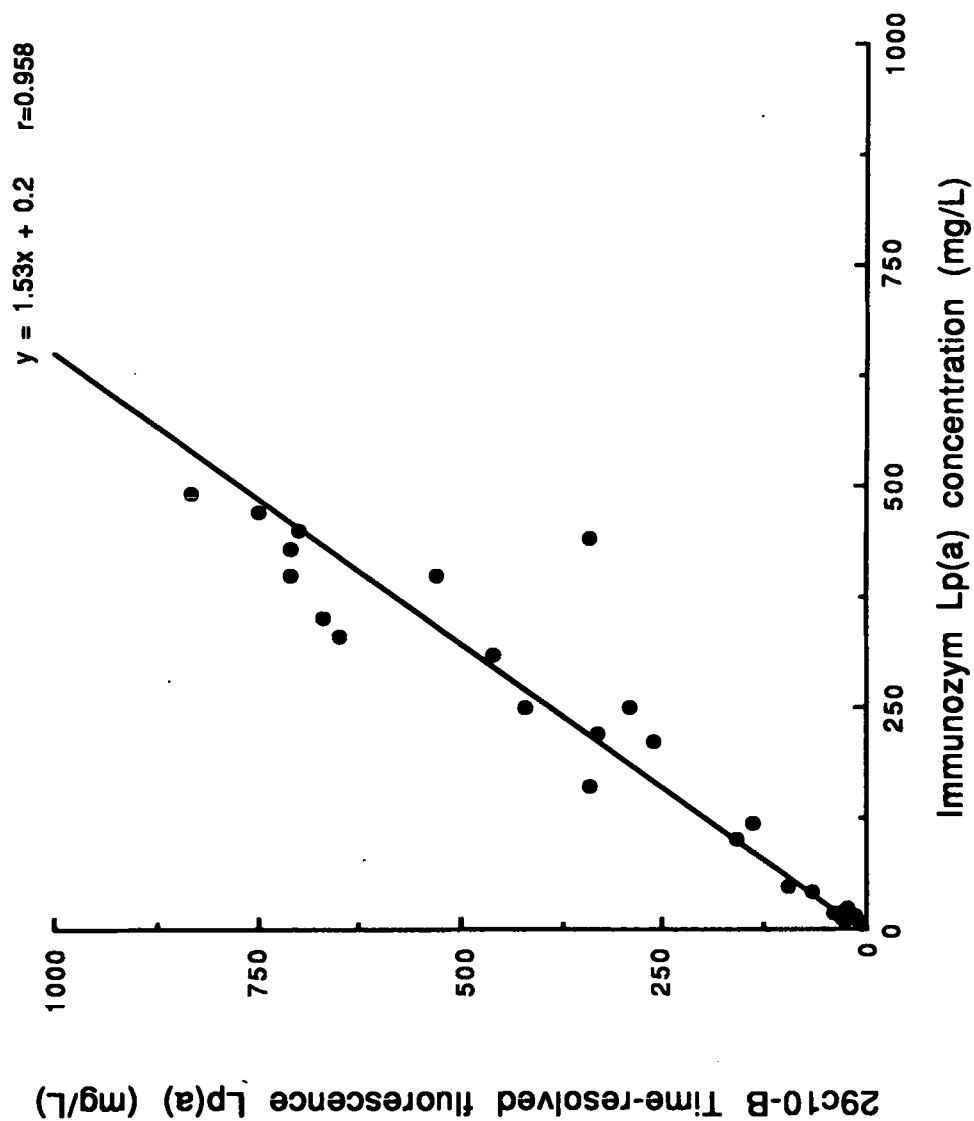
**Figure 6.4**

Performance of the time-resolved fluorescence assay on the Manchester EQAS scheme. Result expressed as standard deviations from the mean of results from all methods.

### 6.5.2 Comparisons with a commercial kit assay.

The Immunozy<sup>®</sup> ELISA assay was used to measure Lp(a) in some research projects undertaken by this department. Human serum samples analysed using this kit were also analysed using the 29c10-B time-resolved fluorescence assay. Quality control samples were analysed in both assays and the values obtained were within acceptable analytical ranges.

Figure 6.5 compares results obtained in the two assays using the same standard. Although the number of data points is small, it is apparent that the time-resolved fluorescence assay generates results up to 50% higher than the Immunozy<sup>®</sup> assay. The reason for the differences in results is probably due to the antibody combinations used in the assays, the Immunozy<sup>®</sup> assay uses two anti-apo (a) antibodies while the 29c10-B assay uses one anti-apo (a) and one anti-apo B antibody. A publication from Immuno<sup>(215)</sup> compared their commercial (a)-(a) ELISA assay with an ELISA (of similar design) using an anti-apo (a) detection antibody in place of the anti-apo B detection antibody. The results they obtained for this (a)-B assay were ~40% higher than for the (a)-(a) assay, this agrees well with comparison data from the 29c10-B assay and the Immunozy<sup>®</sup> ELISA.



**FIGURE 6.5**

A comparison of Lp(a) results obtained for 40 serum samples analysed on the 29c10-B time-resolved fluorescence and Immunozygm ELISA assays.

## **6.6 COMPARISONS BETWEEN DIFFERENT 'IN-HOUSE' TIME-RESOLVED FLUORESCENCE ASSAYS.**

### **6.6.1 Method.**

A series of 24 human serum samples were analysed using microtitre plates coated with one of the three different anti-apo.(a) capture antibodies ('Immuno' anti-apo (a), and the 35f5 & 29c10 monoclonal antibodies) together with the europium labelled anti-apo B detection antibody. The assays were performed on the same day using the same standards. Quality control materials, assayed in each batch, were within acceptable analytical limits.

### **6.6.2 Results.**

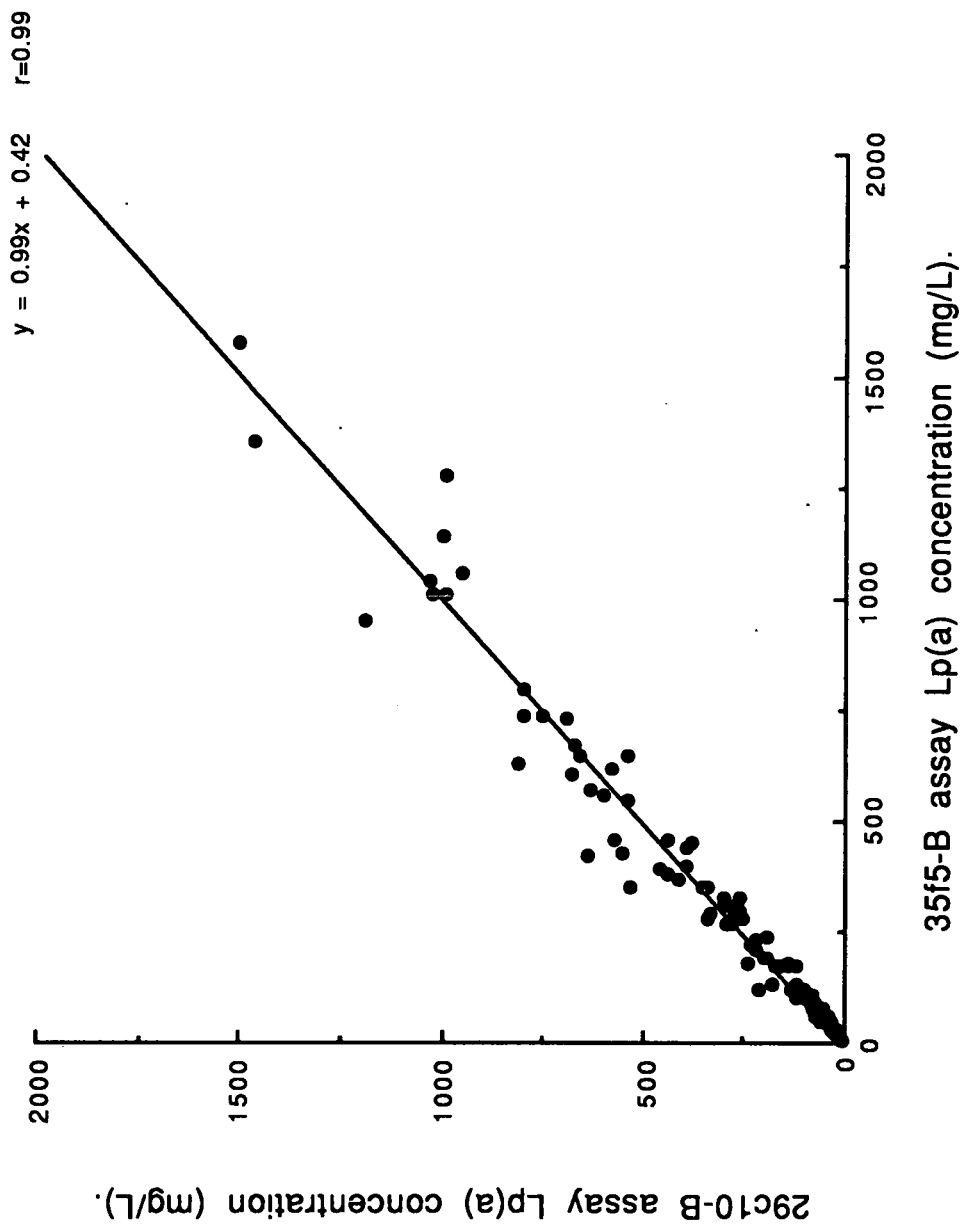
The results for the samples obtained using each antibody combination are presented in table 6.7. It may be seen that 21/24 samples showed good agreement. However, the other three (samples 1, 4 & 9) gave discrepant results. As the same detection antibody was used in each case, this strongly suggests that the capture antibodies have different affinities for the isoforms of Lp(a) in these samples.

A larger study was performed in which results from two Lp(a) assays using a monoclonal anti-apo (a) capture antibody and a polyclonal anti-apo B detection antibody (35f5-B & 29c10-B) were compared. These comparisons are presented in figure 6.6. This plot demonstrates that for a large number of samples the correlation between the two assays is excellent.

**Table 6.5.**

A comparison of Lp(a) results (in mg/L) for 24 serum samples measured using three different time-resolved fluorescence assays.

Sample number	Antibody combination		
	(a)-B	35f5-B	29c10-B
1	75	120	210
2	470	390	450
3	550	440	550
4	260	420	650
5	910	950	1190
6	13	12	16
7	62	55	68
8	360	270	280
9	320	460	920
10	62	53	67
11	320	280	340
12	120	130	180
13	92	100	130
14	35	36	44
15	54	54	50
16	140	180	240
17	560	610	650
18	160	290	330
19	440	460	440
20	480	560	600
21	280	330	300
22	190	210	220
23	70	85	80
24	17	19	20



**FIGURE 6.6** A comparison of Lp(a) concentrations in 112 serum samples measured using two time-resolved fluorescence assays.

## **6.7 INTERFERENCE.**

### **6.7.1 Methods.**

Assay interference resulting from biomolecules present in body fluids has long been recognised. Interferences may be due to pigments that affect colourimetric measurements or, more specifically, cross-reactivity between molecules. The measurement of Lp(a) presents a particular interference problem in that the structure of Lp(a) is homologous to other serum proteins such as apolipoprotein B (which is present in large quantities in lipoproteins such as LDL) and plasminogen (which has a structure similar to apolipoprotein (a)).

To investigate the possibility of interference in Lp(a) assays serum samples with known Lp(a) concentrations were 'spiked' with test compounds obtained commercially. The test agent was added to a final concentration in excess of the highest concentration found in human serum.

### **6.7.2 Results.**

#### **Plasminogen interference.**

The effect of plasminogen on three Lp(a) assays was investigated. In theory, because of the structural similarities between apo (a) and plasminogen, the assays most likely to be affected by plasminogen are those using two anti-apolipoprotein (a) antibodies.

*29c10 - B assay* : Plasminogen was added to two serum samples, with high or low concentrations of Lp(a), to a final concentration of 99, 186 and 372 mg/L (in addition to the endogenous plasminogen concentration). Samples were then analysed in triplicate. A plot of plasminogen added versus measured Lp(a) concentration (figure 6.7) shows that addition of plasminogen at concentrations above those seen in normal human serum <sup>(240)</sup> has no effect on measured Lp(a) concentrations.

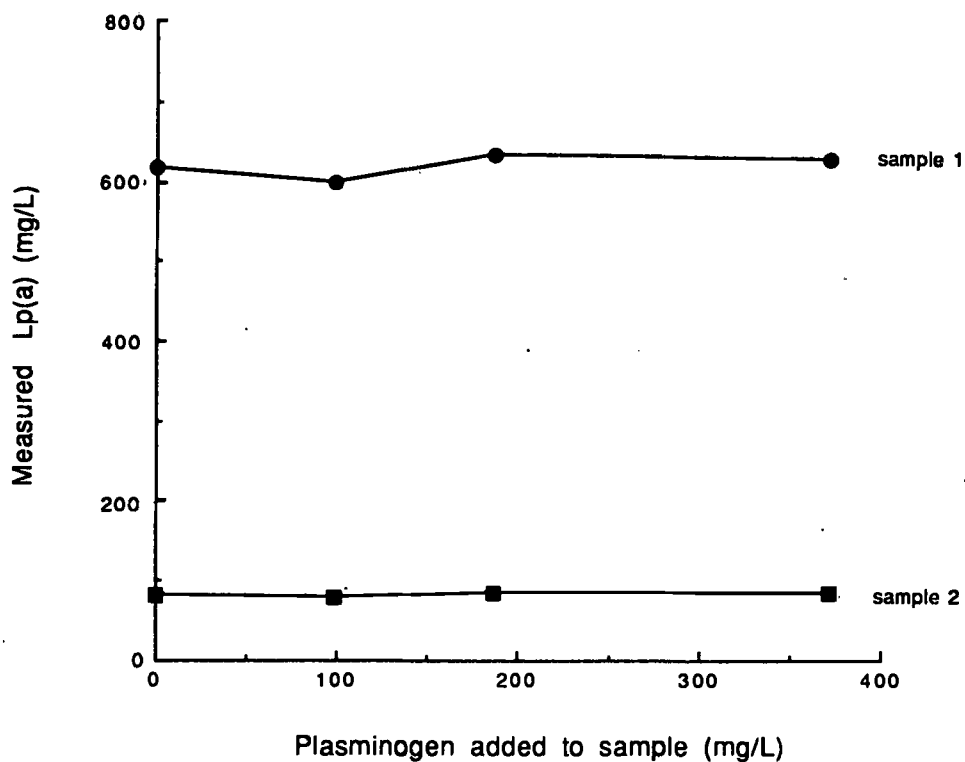


*(a)-(a)* , *35f5-(a)* & *35f5-29c10 assays* : Plasminogen was added to a serum sample with a low concentration of Lp(a) to final concentrations of 70, 140 and 279 mg/L (in addition to the endogenous plasminogen concentration). Samples were then analysed in duplicate. A plot of plasminogen added versus measured Lp(a) concentration (figure 6.8) again shows that addition of plasminogen at concentrations above those seen in normal human serum <sup>(240)</sup> has no effect on measured Lp(a) concentrations.

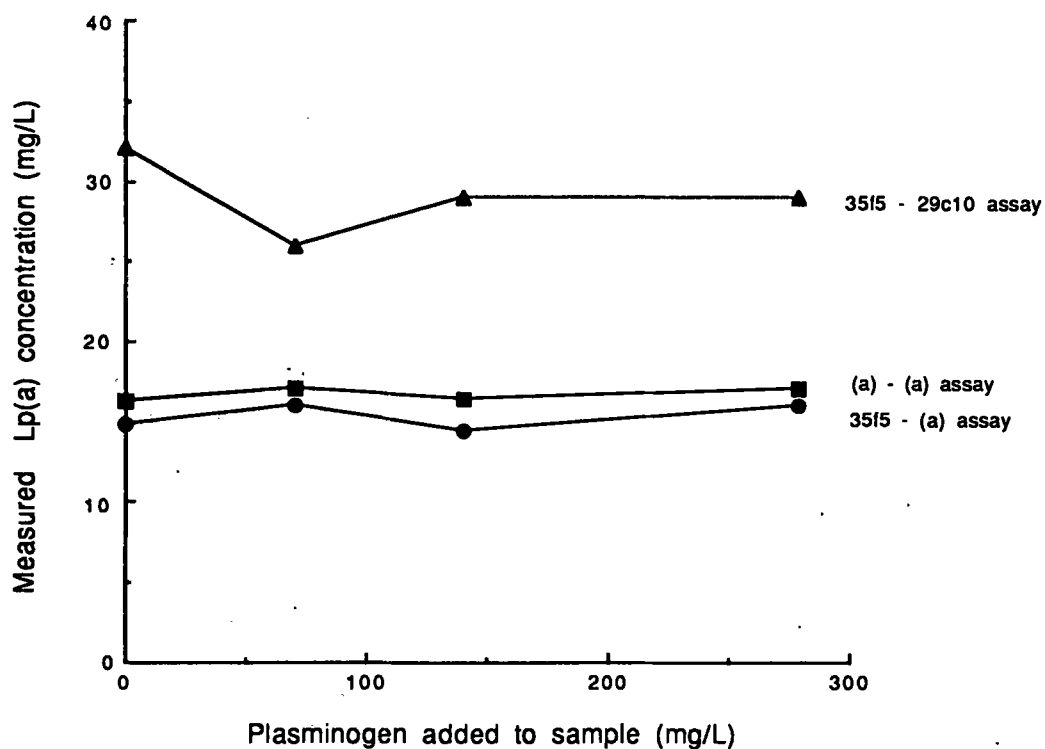
None of the antibody combinations that were tested are affected by high concentrations of plasminogen. The reasons for this may be different in each case.

In two-site assays employing an anti-apo B detection antibody, binding of plasminogen to the detection antibody should not occur. Therefore the only possible mode of interference would be competition between plasminogen and Lp(a) for binding sites on the anti-apo (a) antibody coated plates. Since these binding sites (even if they bound plasminogen) are present in great excess, it was unlikely that interference could occur.

Assays using two anti-apo (a) antibodies were most likely to be susceptible to plasminogen interference. There is evidence (chapter 5) that the in-house monoclonal antibodies are directed against the kringle 4 domains of apo (a). These domains are present in plasminogen , but the antibodies do not appear to cross-react.



**Figure 6.7** The effect of increasing plasminogen concentration measured Lp(a) using the 29c10-B time-resolved fluorescence Lp(a) assay.



**Figure 6.8** The effect of increasing plasminogen concentration on measured Lp(a) using time-resolved fluorescence assays with two anti-apo (a) antibodies.

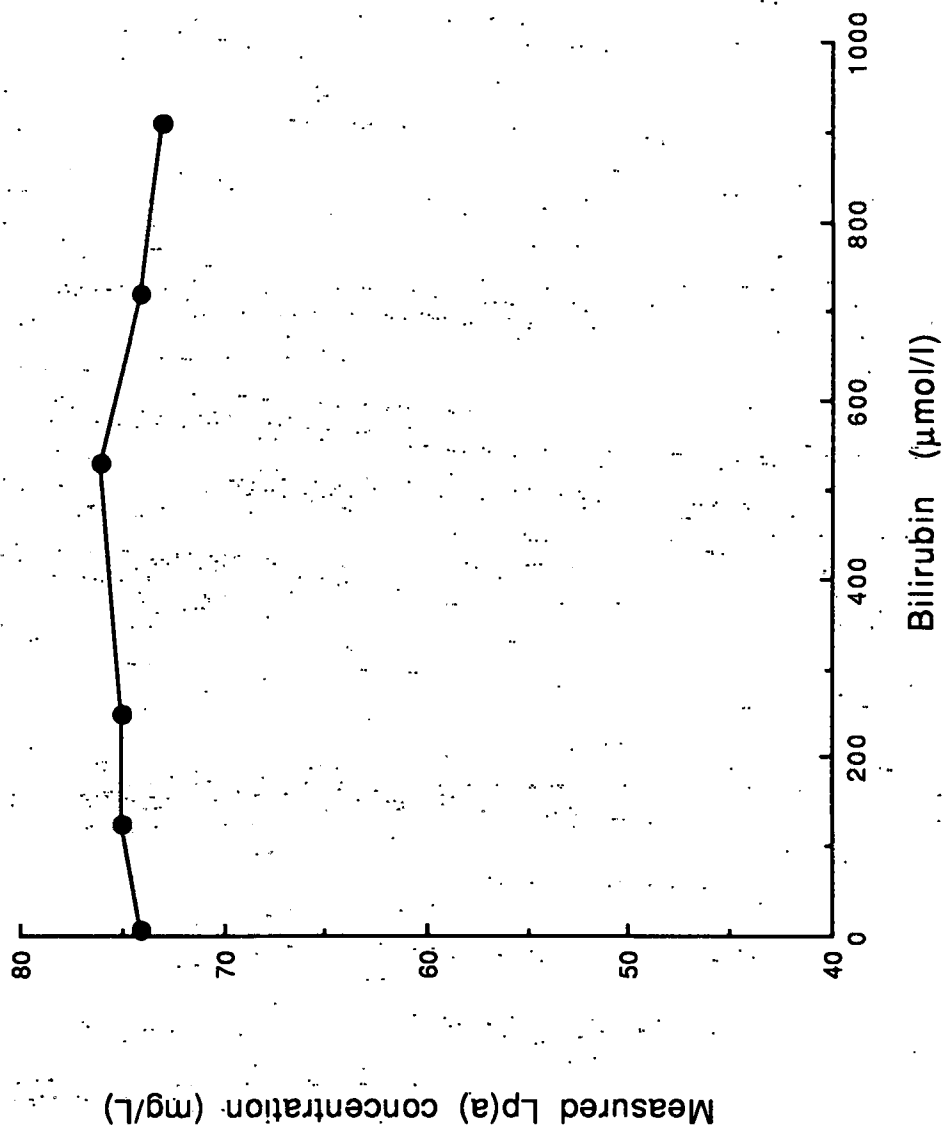
### **Bilirubin interference.**

Bilirubin was investigated as a possible interferent since it was known to quench fluorescence under certain conditions <sup>(241)</sup>. Bilirubin was added to serum samples over the range 120-900  $\mu\text{mol/L}$ . The final bilirubin concentrations in each sample (estimated on the DuPont Dimension AR<sup>®</sup> autoanalyser) were 6, 124, 248, 531, 720 and 908  $\mu\text{mol/l}$ . The samples were then assayed for Lp(a), in triplicate, using the 29c10-B assay. A plot of bilirubin concentration versus measured Lp(a) concentration (figure 6.9) indicates that at concentrations up to 50 times those found in human serum <sup>(242)</sup> bilirubin had no effect on measured Lp(a) concentrations.

Other possible interferents have not been investigated. Although it is likely that the specificity of a two-antibody procedure coupled with a large sample dilution would prevent interference from other biomolecules, this possibility has not yet been investigated. LDL and other apo B-containing proteins may be a problem in a one-step (a)-B assay due to competition for the detection antibody. In the two-step assay format used in this project the possibility of interference from these proteins is unlikely because apo B should not bind to the anti-apo (a) capture antibody.

### **CONCLUSIONS.**

The data shows that the time-resolved fluorescence assay is superior in terms of sensitivity, precision and working range to any of the commercial assays. Comparisons of results for serum samples with those obtained from the commercial kits showed considerable variability which is probably due to the different antibody combinations and standards used in the assays. The only method by which comparability of measured Lp(a) results may be achieved is by the use of the same combination of antibodies and the same standard material containing a mixture of Lp(a) isoforms, although this is not likely to happen in the foreseeable future.



**Figure 6.9** The effect of increasing bilirubin concentrations on measured Lp(a) using the 29c10-B time-resolved fluorescence assay.

**CHAPTER 7**

**CLINICAL MEASUREMENTS USING THE DELFIA® Lp(a) ASSAY**

## **7.1 ESTABLISHMENT OF A 'REFERENCE RANGE' <sup>(243)</sup> for SERUM Lp(a).**

### **7.1.1 Method.**

200 serum samples (98 from females, 102 from males) were analysed for Lp(a) using the 29c10-B assay. The samples were obtained from healthy Caucasian blood donors. Serum was frozen at -20°C on the day of venepuncture and stored for three months at the same temperature before analysis. The age of the subjects ranged from 17 to 67 years.

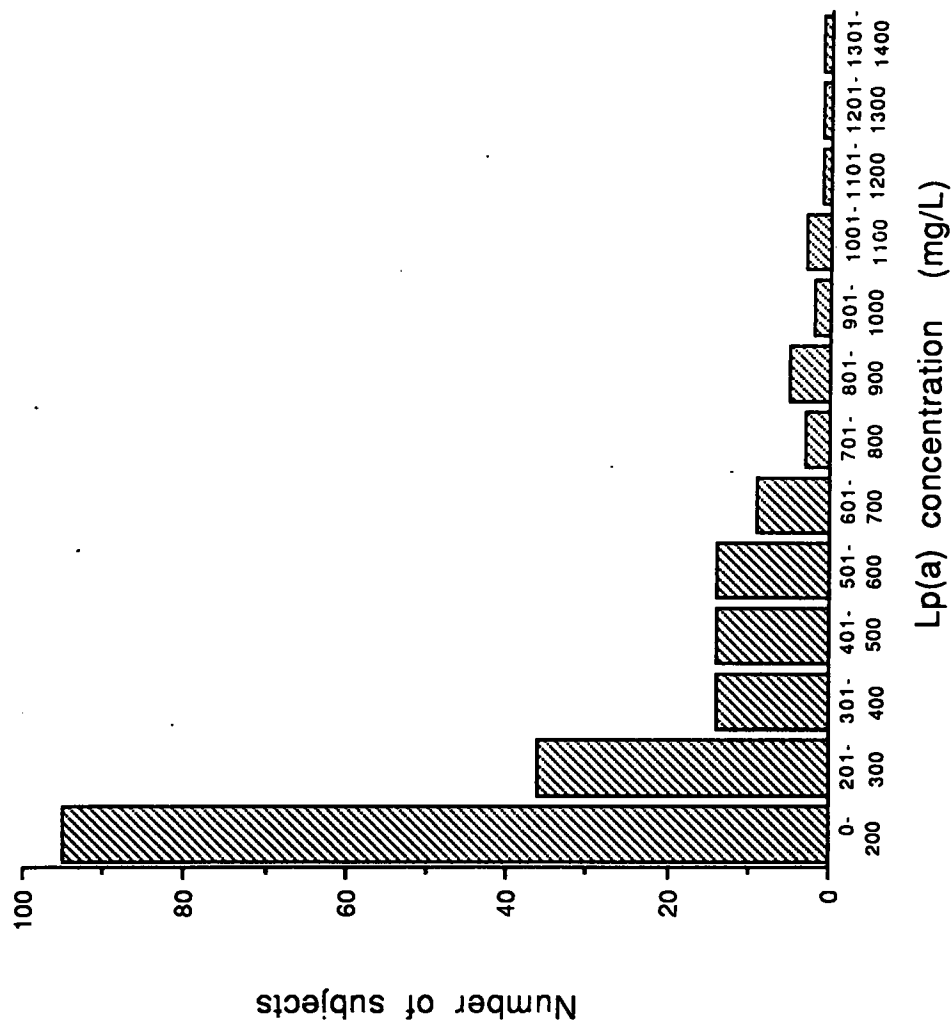
### **7.1.2 Results.**

#### ***Population distribution of serum Lp(a) concentrations.***

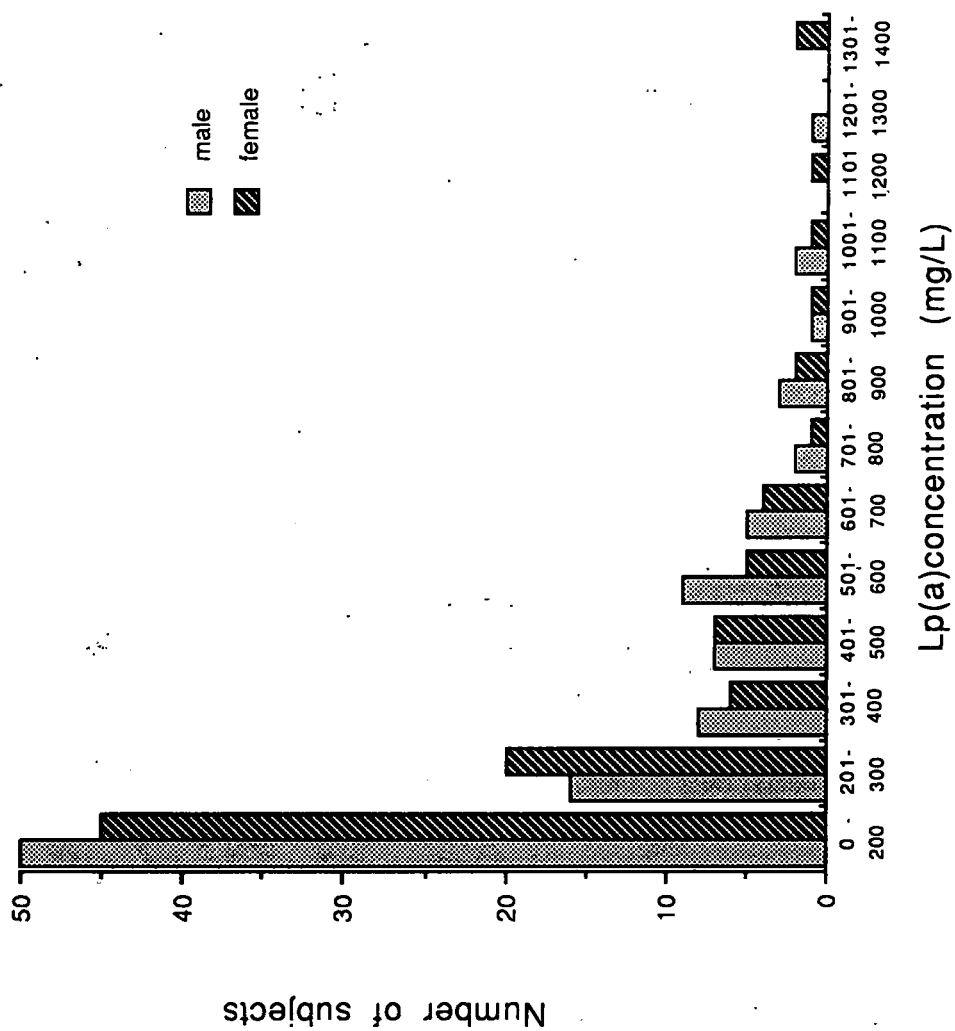
Figure 7.1 shows the distribution of serum Lp(a) concentrations for the 200 adults. Lp(a) was detectable in all of the samples assayed: the lowest concentration was 4 mg/L and the highest was 1380 mg/L. Lp(a) was also measured in a further one hundred hospital in-patient serum samples each of which had concentrations of Lp(a) well above the detection limit (1 mg/L) of the assay (data not shown). This strongly suggests that Lp(a) is normally present in the serum of healthy Caucasian adults.

#### ***Comparison of serum Lp(a) concentrations in males and females.***

Figure 7.2 shows a comparison of the distribution of serum Lp(a) concentrations in males with those in females: the male group had a mean concentration of 210 mg/L (median of 120 mg/L) while the female group had a mean of 236 mg/L (median of 110 mg/L). This data suggests that there is no significant difference in serum Lp(a) concentrations between healthy adult Caucasian males and females. The findings are consistent with previous studies in which sex-independent, highly skewed distributions of serum Lp(a) concentrations were reported for the Caucasian population <sup>(43)</sup>.



**Figure 7.1** Distribution of serum Lp(a) concentrations in 200 healthy adult Caucasian subjects.



**Figure 7.2** Distribution of serum Lp(a) concentrations in a group of 200 healthy adult males and females.



Many clinically useful analytes (e.g. serum potassium) show a Gaussian (bell-shaped) distribution in the healthy population. It is accepted procedure <sup>(243)</sup> that, for this type of distribution, subjects with analyte concentrations between  $\pm 2$  standard deviations of the mean concentration are deemed 'normal'. Statistically, this figure represents 95% of a normally distributed population, i.e. 1 in 20 of the healthy population have analyte concentrations outside this 'normal' range. This statistical analysis does not apply to skewed analyte distributions (as presented here for Lp(a)). However, logarithmic transformation of data from skewed distributions may produce a Gaussian type curve <sup>(243)</sup>. Unfortunately, however, logarithmic transformation of the highly skewed Lp(a) data does not generate such a curve.

Other strategies for establishment of a Lp(a) reference range were therefore investigated. The percentage of the population with Lp(a) concentrations below certain values was determined:- 3.5% had a serum Lp(a) concentration below 10 mg/L, 46% had a concentration below 100 mg/L, 73% had a concentration below 300 mg/L, 87% had a concentration below 500 mg/L and 95% of the population had serum Lp(a) concentrations below 780 mg/L. From this data it is obvious that 95% confidence limits cannot be used to establish a 'reference range', mainly because only patients with the most elevated of Lp(a) concentrations would be classified as abnormal. Current views <sup>(60)</sup> are that Lp(a) concentrations above 300 mg/L are associated with an increased risk of coronary artery and other vascular diseases. This limit was determined by retrospective clinical assessment of patients with coronary heart disease whose Lp(a) was measured using assays employing two anti-apo (a) antibodies. In this project (see chapter 6) and other studies <sup>(215)</sup> assays using one anti-apo (a) and one anti-apo B antibody gives results that are ~40% higher than those using two anti-apo (a) antibodies. Hence, it may be that a discriminatory value of 420 mg/L should apply when assays using this antibody combination are employed.

Data presented in chapter 6 has examples of serum samples which produced a wide range of results in assays employing several different antibody combinations. For example, one sample gave results varying from 360-890 mg/L. Such wide

analytical variability makes clinical interpretation of Lp(a) results very difficult. It may be that only patients with Lp(a) concentrations below 200 mg/L, and with no other associated coronary risk factors, may be classified as having a low risk of coronary artery disease.

## 7.2 AGE-DEPENDENT VARIATIONS IN SERUM Lp(a) CONCENTRATION.

Serum from 200 healthy Caucasian adults, 62 children and 21 samples of cord blood were analysed using the 29c10-B assay. The paediatric samples were obtained from hospital in-patients with an age range from one week to 15 years. The cord blood was collected following uncomplicated delivery.

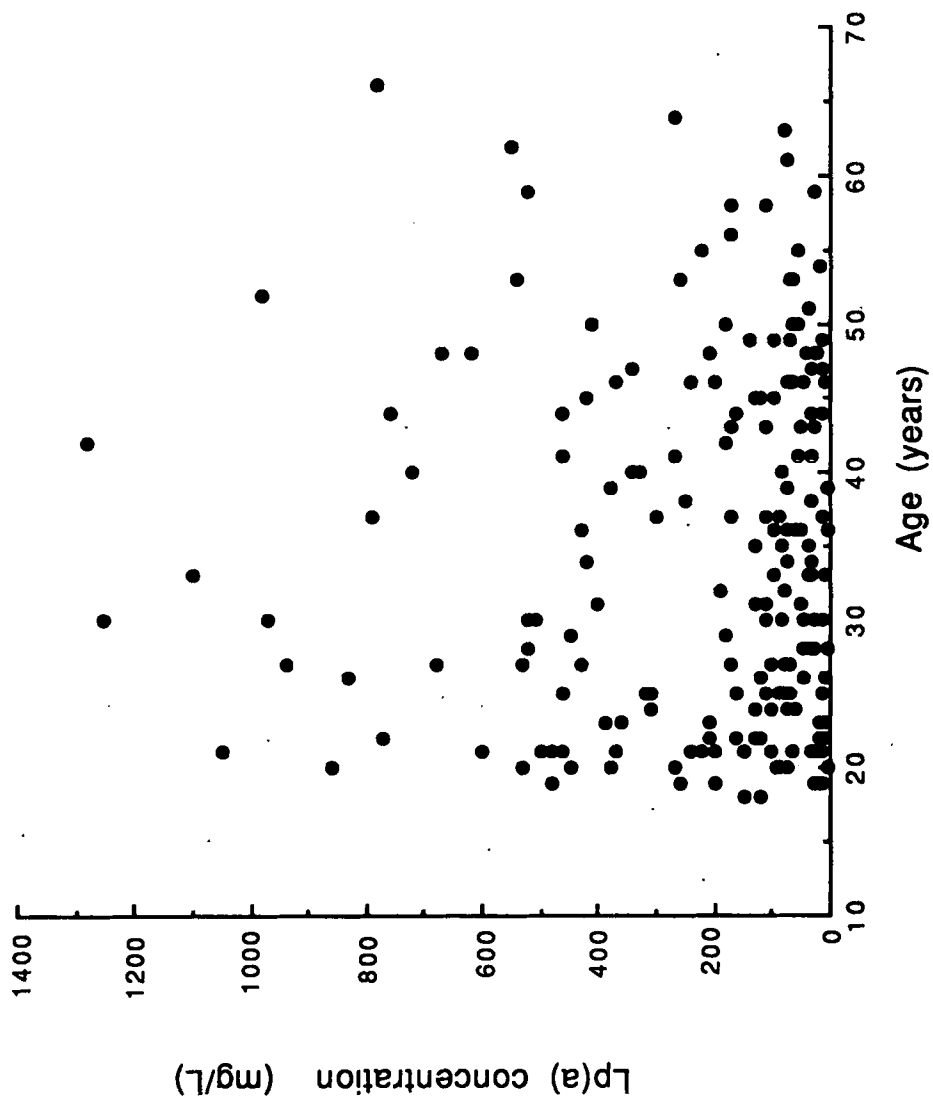
### 7.2.1 Results.

**Adults :** The distribution of serum Lp(a) concentrations with age in the 200 healthy Caucasian adults is shown in figure 7.3. It is apparent that there is no correlation between age and serum Lp(a) concentration.

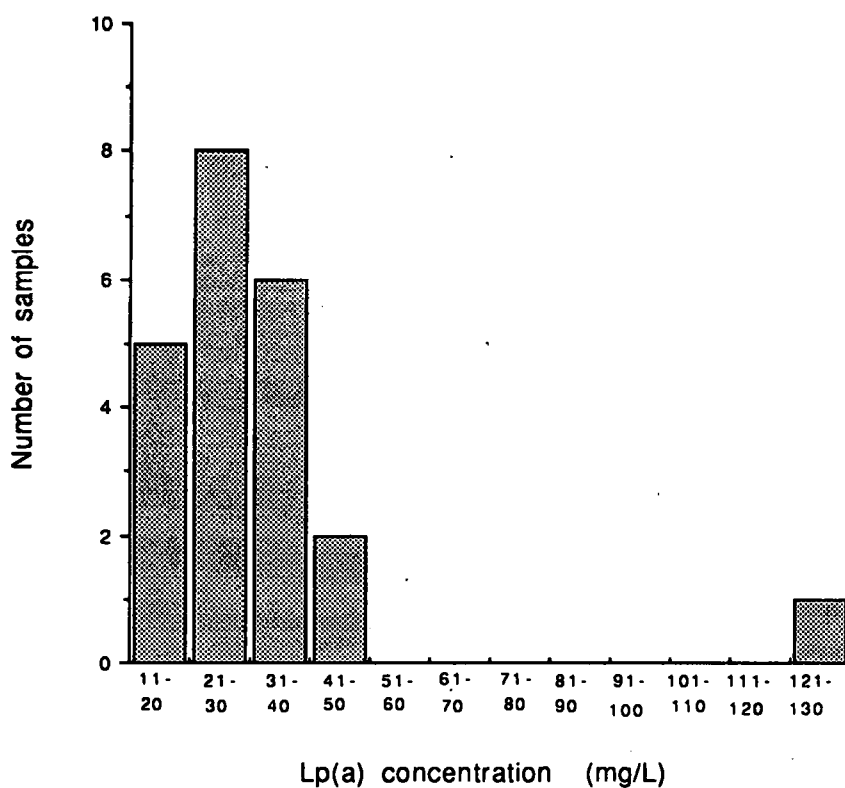
**Cord blood :** The distribution of Lp(a) concentrations is presented in figure 7.4. Twenty samples had serum Lp(a) concentrations below 40 mg/L whereas a single sample had a concentration of 110 mg/L. All samples were found to have measurable concentrations of Lp(a), the lowest of which was 1 mg/L.

**Children :** The distribution of serum Lp(a) concentrations is shown in figure 7.5. The shape of the distribution pattern was similar to that obtained for adults (fig 7.3), although the Lp(a) concentrations were lower. As with adults there was no obvious correlation between age and Lp(a) concentration (data not shown). However, this group of children could not be regarded as 'normal' because many of the subjects were hospitalised due to a metabolic disorder or were receiving parenteral nutrition.

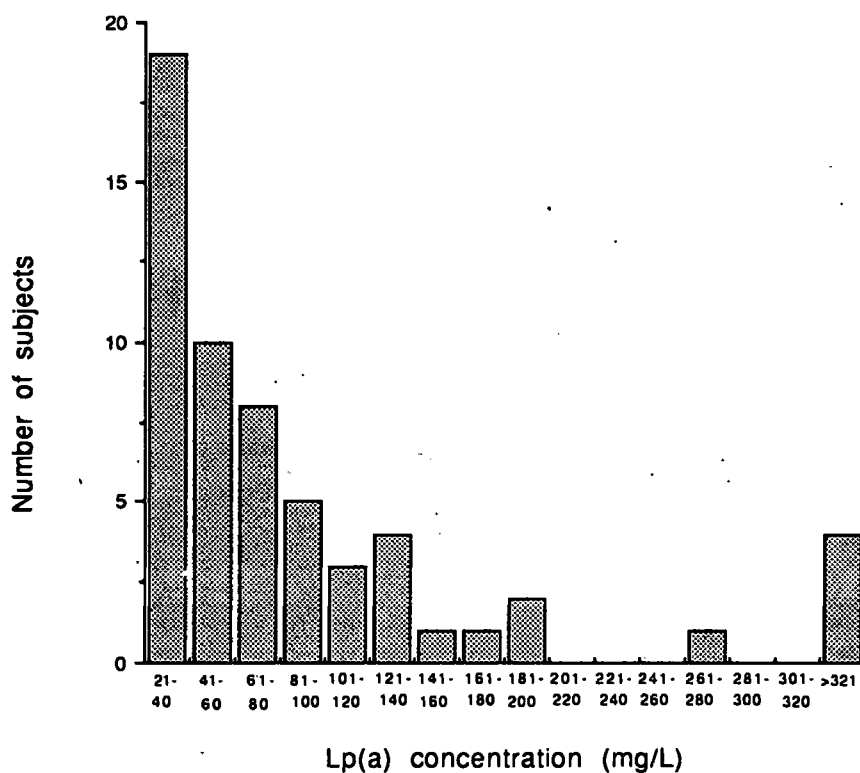
These findings are consistent with previously published data. No correlation has ever been made between serum Lp(a) concentrations and age in healthy Caucasian adults <sup>(33)</sup>. The finding of low Lp(a) concentrations during the neonatal period has also been documented <sup>(49)</sup>. Various studies <sup>(43-45)</sup> have suggested that adult levels of Lp(a) are attained by late childhood and thereafter remain constant. However, in the absence of a long term research investigation into healthy juvenile serum Lp(a) concentrations, this is difficult to confirm.



**Figure 7.3** Serum Lp(a) concentrations versus age in a group of 200 healthy adults.



**Figure 7.4** Distribution of Lp(a) concentrations in 22 samples of cord blood.



**Figure 7.5**  
Distribution of serum Lp(a) concentrations in a group of 62 hospitalised children:

### **7.3. TIME-DEPENDANT VARIATION IN SERUM Lp(a) CONCENTRATIONS.**

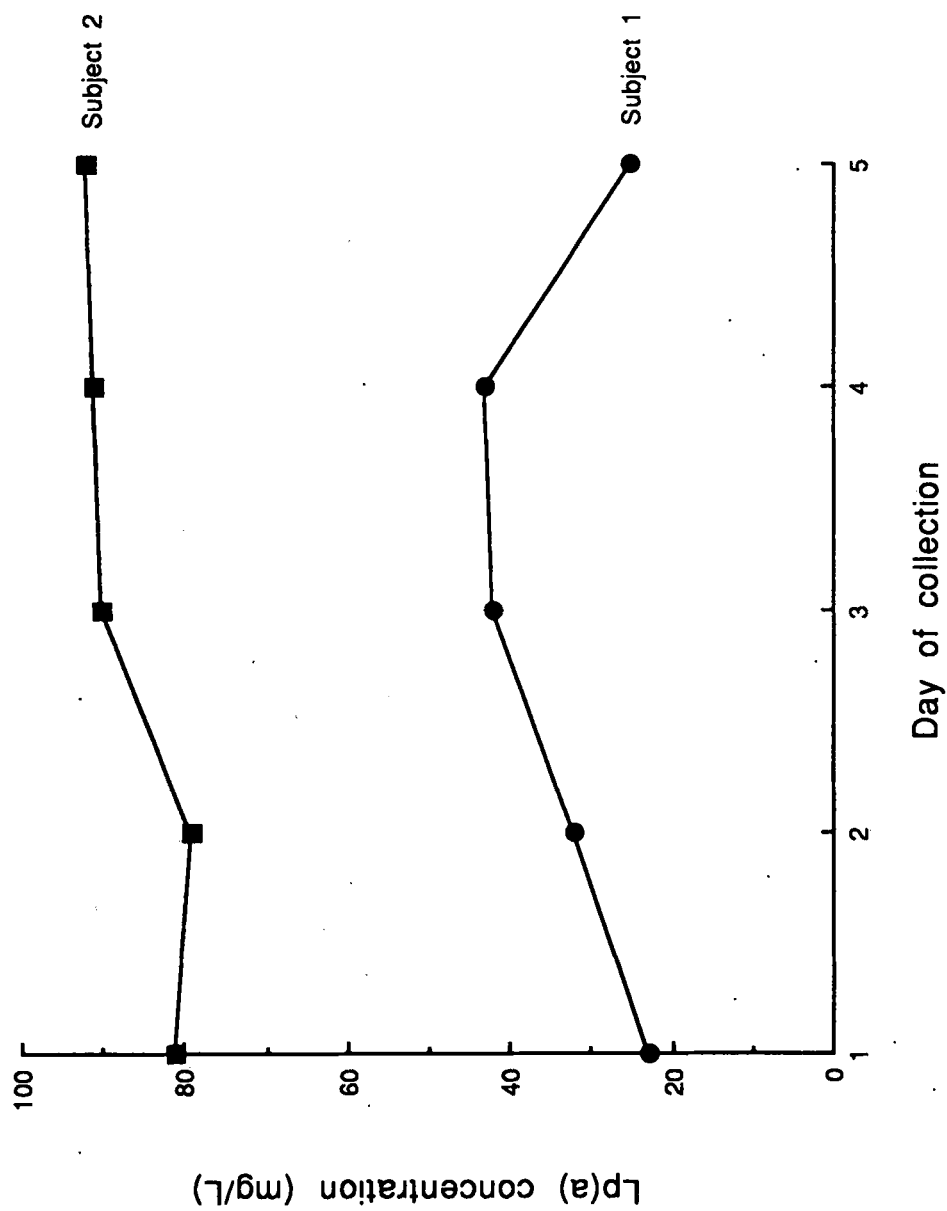
#### **7.3.1 Method.**

Blood samples were collected from two healthy female volunteers (aged between 20-30 years) on five consecutive days. The subjects were fasted overnight and blood was collected at 9 a.m. the following day. The blood was centrifuged and the serum was separated and frozen at -20°C. All 5 samples were stored for 2 weeks then thawed, centrifuged and analysed (in duplicate) using the same 29c10-B assay. Quality control samples assayed with the batch fell within acceptable analytical limits.

#### **7.3.2 Results.**

The serum Lp(a) concentrations for each of the subjects are shown in figure 7.6. Both subjects had low serum Lp(a) concentrations. However, since the assay is very precise at these concentrations (coefficient of variation of 6% at a concentration of 40 mg/L) it is unlikely that the large percentage variations in Lp(a) concentration seen for subject 1 was due to assay imprecision.

The possibility that venous stasis during blood collection may have artefactually elevated the serum concentrations of large molecules (such as proteins) was investigated. Serum total protein and albumin concentrations (which normally change little from day-to-day in healthy individuals but rise following prolonged venous stasis) were measured using the DuPont Dimension® autoanalyser. Concentrations for subject 1 are presented in table 7.1. Protein concentration variations greater than 10 g/L would indicate significant haemoconcentration.



**Figure 7.6**  
Variations in serum Lp(a) concentrations of 2 subjects over five consecutive days.

**Table 7.1**

Concentrations of three analytes measured in the serum of subject 1 over 5 days.

	Lp(a) mg/L	T.Protein g/L	Albumin g/L
Day 1	23	74	43
Day 2	33	74	43
Day 3	42	69	41
Day 4	43	68	40
Day 5	28	70	41

This data suggests that haemoconcentration was not the cause of the variability of Lp(a) concentration. Unfortunately no further volunteers were recruited, but further studies into this large percentage variation (particularly if it is repeated at high concentrations of Lp(a)) might prove to be very important to the interpretation of Lp(a) measurements.

#### **7.4. DISCUSSION.**

Data from the short clinical studies presented in this chapter is in close agreement with previously published findings. These findings confirm that the time-resolved assay performs in a similar manner to the commercially available kit assays used in other studies.

Many studies have been performed in recent years in which serum Lp(a) concentrations have been measured in various disease states, and following drug therapy. A collaborative study with consultant cardiologist, Dr P. Weissberg, at Addenbrooke's Hospital has recently begun. The aim of the study is to screen patients attending the hospital lipid clinic to identify those with raised serum Lp(a) concentrations and to assess the effectiveness of a drug therapy aimed at lowering serum Lp(a) concentrations in volunteers from this group. The project is, at yet, only at the screening stage.



**CHAPTER 8**  
**EFFECT OF DETERGENTS ON SERUM Lp(a) MEASUREMENTS**

## 8.1 INTRODUCTION.

It has been suggested that Lp(a) has a tendency to self-associate (i.e. aggregate), particularly following freeze-drying procedures <sup>(211)</sup>. If such self-association also occurs *in vivo*, it is possible that Lp(a) concentrations measured by immunoassay might not reflect the true serum Lp(a) concentration.

Detergents are commonly used to dissociate large insoluble lipid aggregates into smaller, more soluble, micelles. The effect of adding different detergents to the buffers used in the time-resolved fluorescence assay for Lp(a) was therefore investigated.

Three different types of detergent are available <sup>(244)</sup>.

(i) *Ionic Detergents* are molecules consisting of a hydrocarbon chain and a strongly acidic or basic polar group. An example of this class of detergent is sodium dodecyl sulphate (SDS). Ionic detergents frequently cause denaturation of proteins in solution.

(ii) *Non-Ionic Detergents* are molecules consisting of a hydrocarbon chain with a polyoxyethylene polar region. They rarely cause structural damage to proteins and consequently are frequently incorporated into assay buffers. These detergents are particularly useful in immunoassays, where the addition of small amounts of detergent to the reaction mixture may significantly reduce non-specific binding of labelled antibodies.

A wide range of non-ionic detergents are commercially available with molecular weights ranging from 500-2000 Daltons. Examples include the 'Triton' (Alkylphenyl Polyoxyethylene ether) and 'Tween' (Acyl polyoxyethylene ether) series.

(iii) *Bile salts (anionic detergents)* consist of a steroid ring structure with hydroxyl groups located on the outside of the molecule and an ionic group at one end. This structure is completely different to the other two types of detergent and, consequently, the physical structure of micelle formation is different to those formed with ionic and non-ionic detergents. Examples of bile salts are cholic acid and deoxycholic acid.

The assay buffer (Tris HCl) provided with commercial DELFIA® kits contains the detergent Tween 20 at a concentration of 0.02% (v/v). When serum samples were analysed following dilution in this buffer, or in Tween 20-free Tris-HCl buffer, there was no difference in the fluorescence signals obtained (data not shown). This suggests that the low concentration of detergent had little effect on the Lp(a) assay. DELFIA® assay buffer was therefore used for dilution of serum samples in this study, with addition of other detergents as required.

## **8.2 Effect of non-ionic detergents.**

Serum Lp(a) was assayed in microtitre plates coated with either the 'Immuno' polyclonal anti-apo (a) antibody or the monoclonal antibody, 35f5, in conjunction with a europium-labelled anti-apo B detection antibody. Serum samples were diluted 1:1000 in assay buffer containing the non-ionic detergent, Triton X-100, at concentrations up to 10% (v/v). Fresh Triton/buffer solutions were prepared for each assay.

### **8.2.1 Results.**

In the first experiment, a series of randomly selected serum samples were analysed (together with the Lp(a) standard) following dilution in assay buffers containing Triton X-100 at 0-10% (v/v) using an anti-apo (a) coated plate. The standard Lp(a) assay protocol was used (chapter 5, appendix 5.1).

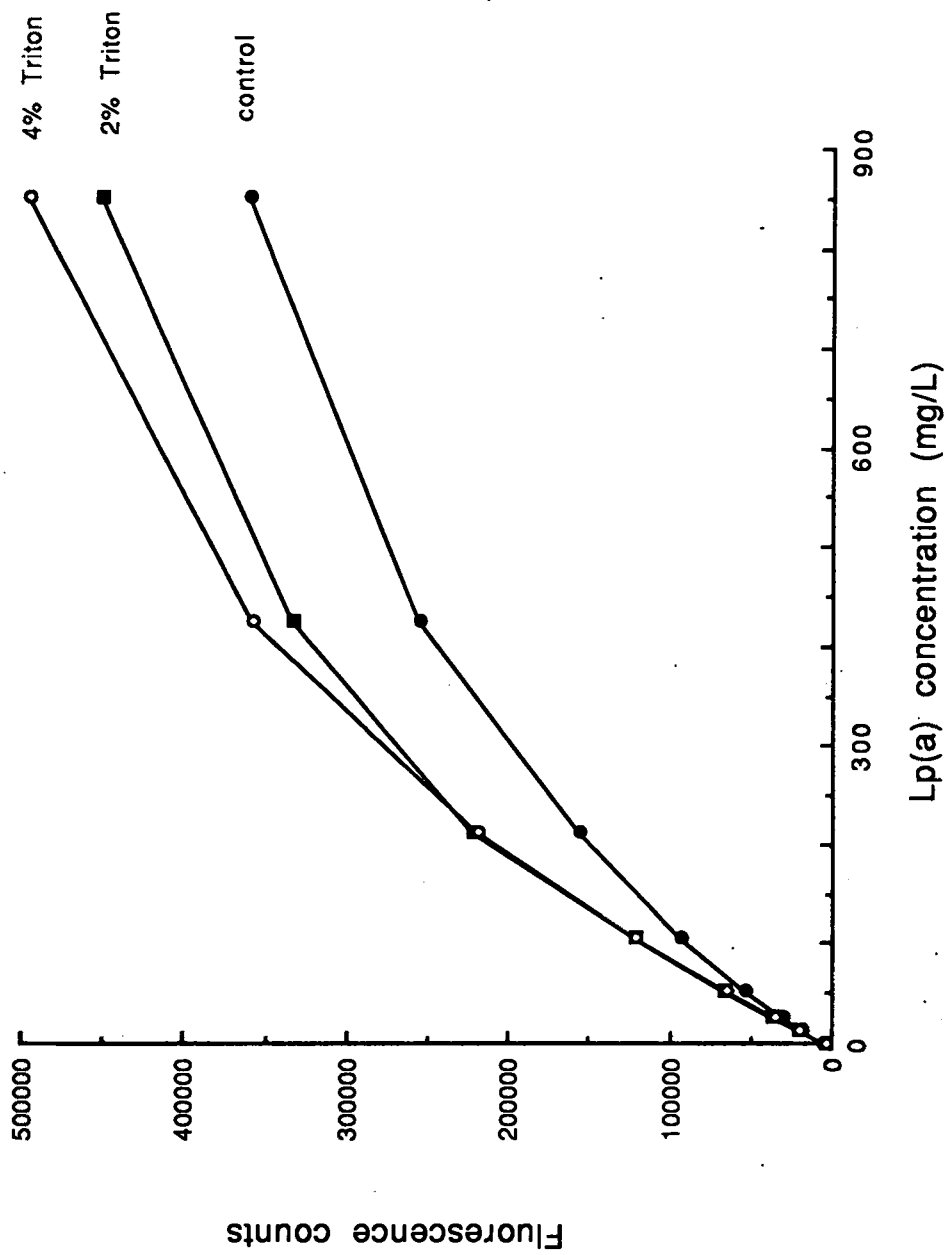
Figure 8.1 shows the dose-response curves for the Lp(a) standard diluted in buffers containing Triton X-100 at 0, 2 and 4% (v/v). Figure 8.2 shows the percentage increase in fluorescence signal (which is proportional to measured Lp(a) concentration) for 8 serum samples assayed after dilution in buffers containing Triton X-100 at 0-10% (v/v). The percentage increase in signal was calculated by comparing the fluorescence counts obtained when the sample was diluted in Triton-free buffer to those obtained when the sample was diluted in detergent-containing buffers.

These data demonstrate that significant increases in fluorescence signal may be obtained when Triton X-100 is added to the assay buffer. Increases were greater than 100% for some of the serum samples. The percentage signal increase does not appear to be related to the Lp(a) concentration of the samples.

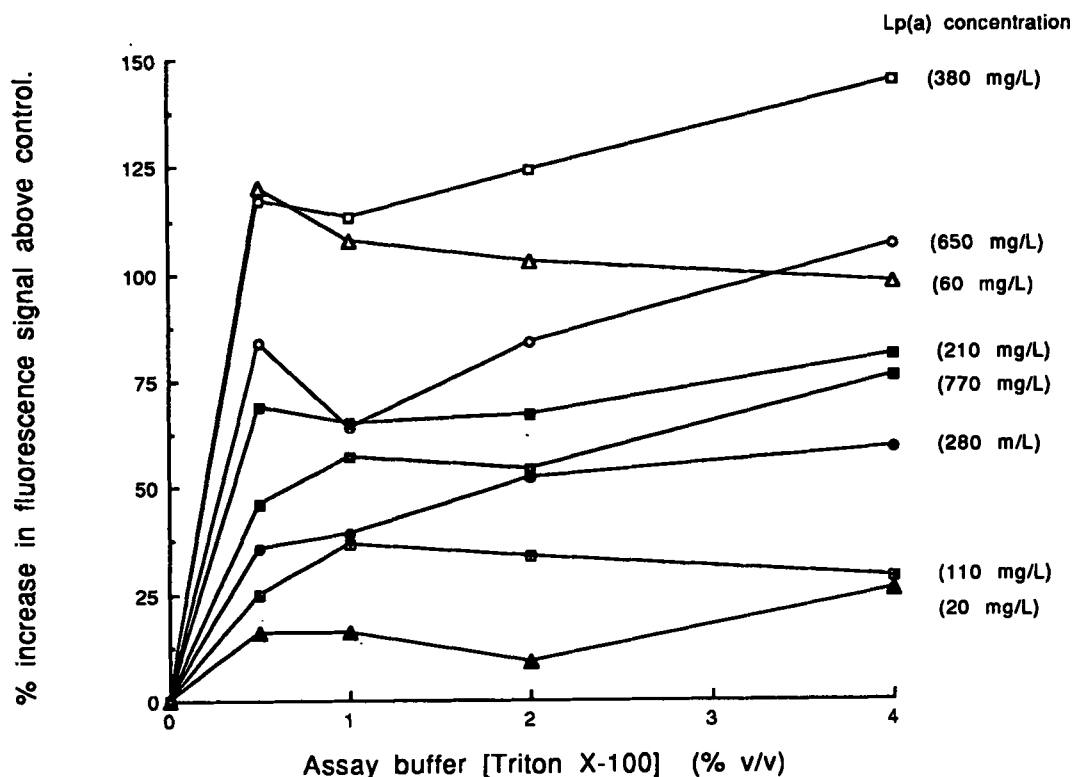
A similar experiment was performed with the same samples using a 35f5 coated plate (results shown in figure 8.3). The percentage increases in signal were less than those obtained using anti-apo (a) coated plates; indeed, two of the eight samples showed a slight decrease in fluorescence signal when Triton X-100 was added to the assay buffer. Assays employing anti-apo (a) antibodies to both capture and detect Lp(a) also showed an increase in fluorescence signal when Triton was added to the assay buffer (data not shown).

### **8.2.2 Discussion.**

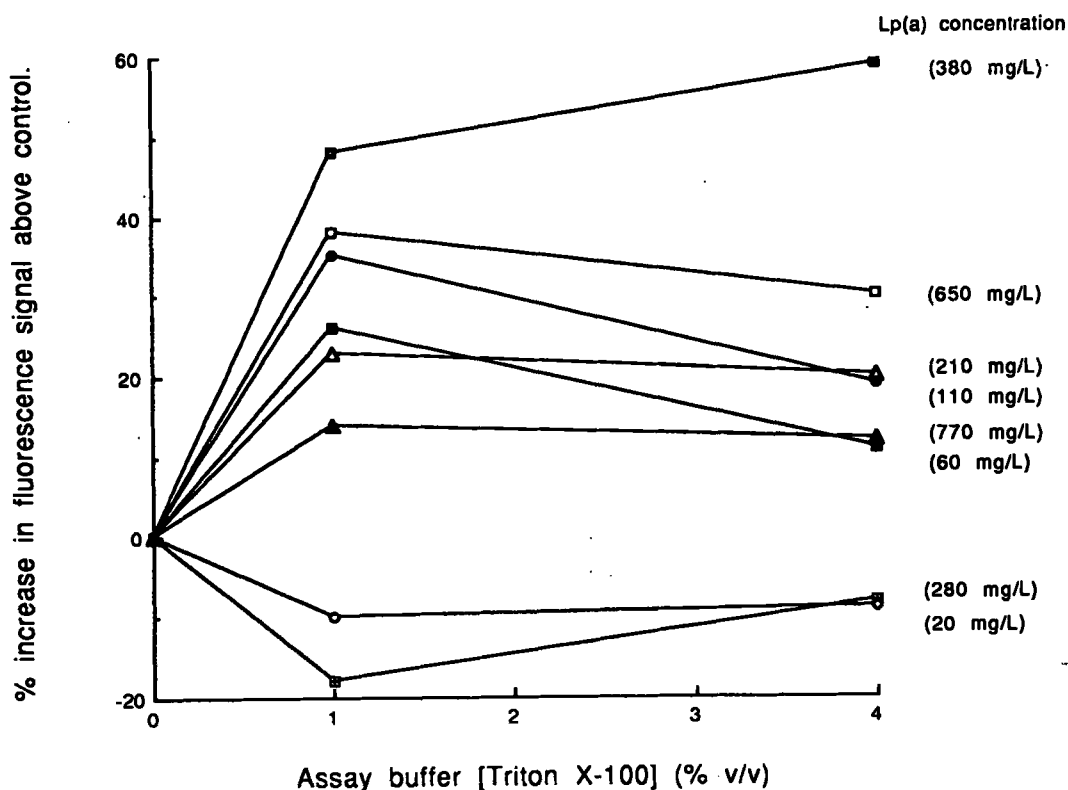
The variable fluorescence signal increases observed with different capture antibodies may be related to their binding to different epitopes on Lp(a). The increases in signal may be explained as follows: if the 'Immuno' polyclonal anti-apo (a) antibody recognised additional epitopes that are usually buried within aggregated Lp(a) particles, then breakdown of the aggregate would cause a marked increase in available antibody binding sites. This would lead to more Lp(a) binding to the plate, which in turn would lead to an increased fluorescence signal. Conversely, if the 35f5 antibody recognised an epitope that is solely on the surface of aggregated Lp(a), then breakdown of the aggregate is less likely to release fewer additional binding sites and, therefore, cause a smaller increase in fluorescence signal. A high proportion of the total increase in fluorescence signal was achieved by increasing the Triton X-100 concentration in the assay buffer from 0 to 1% (v/v). Further addition of Triton produced only a small additional increase in signal.



**Figure 8.1** Lp(a) standard curves assayed using the (a)-B antibody combination and assay buffer containing 0, 2 and 4% (v/v) Triton X-100.



**Figure 8.2** % increase in fluorescence signal when up Triton X-100 (up to 4% v/v) was added to the (a)-B time-resolved fluorescence assay buffer. Increases shown for 8 samples at a range of Lp(a) concentrations.



**Figure 8.3** % increase in fluorescence signal when Triton X-100 (up to 4% v/v) was added to the 35f5-B time-resolved fluorescence assay buffer. Increases shown for 8 samples at a range of Lp(a) concentrations.

### **8.3 Effect of sample storage.**

The possibility that freezing of serum samples causes aggregation of Lp(a) was investigated. A series of 4 serum samples were selected, and a portion of each sample was divided into aliquots and frozen at -20°C, whilst the remainder was stored at 4 °C. Samples were stored at each temperature for 1 day after venepuncture.

One aliquot of each frozen sample was thawed (in air) at 20°C whilst another was thawed in a waterbath at 37°C . Each of these aliquots, along with aliquots of serum stored at 4°C (and subsequently brought to room temperature), were mixed and centrifuged at 20°C. The samples were analysed in assays using the (a)-B or 35f5-B antibody combination after dilution in assay buffer containing 0, 1 or 4 % (v/v) Triton X-100. Standard curves were generated for each of the possible antibody combinations and Triton X-100 concentrations. Results are presented in table 8.1.

It is apparent from the data that:

- (i) All samples displayed some increase in their measured Lp(a) concentrations when Triton was added to the assay buffer.
- (ii) The increase in Lp(a) concentration was greater using the anti-apo (a) coated plates.
- (iii) The method of sample storage or thawing had little effect on the measured Lp(a) concentration. This suggests that freezing of serum does not enhance aggregation of Lp(a).
- (iv) The percentage increase in measured Lp(a) concentration appeared to be independent of Lp(a) concentration (over the range ~40-390 mg/L).

**Table 8.1 : Lp(a) concentrations of 4 serum samples (stored and thawed in three different ways) measured using the (a)-B and 35f5-B assay with buffers containing 0,1 & 4 % (v/v) Triton X-100.**

**(a)-B assay.**

Sample	Stored at -20°C						Stored at +4°C		
	Thawed at 20°C			Thawed at 37°C					
	0%	1%	4%	0%	1%	4%	0%	1%	4%
1	190	290	340	160	280	330	150	290	330
2	390	880	920	380	850	950	370	750	830
3	40	60	70	50	70	70	40	60	70
4	250	540	610	280	560	600	250	530	600

**35f5 -B assay.**

Sample	Stored at -20°C						Stored at +4°C		
	Thawed at 20°C			Thawed at 37°C					
	0%	1%	4%	0%	1%	4%	0%	1%	4%
1	270	270	300	220	280	300	220	280	290
2	660	820	1040	640	810	980	620	820	950
3	50	60	70	70	70	80	50	60	70
4	520	610	670	480	620	680	500	620	680



The observed increase in measured Lp(a) concentration when serum samples and standards were assayed in buffers containing Triton X-100 suggests that the serum samples gave a greater increase in signal than the Lp(a) standard. If both signals had increased proportionately then the measured Lp(a) concentration in the serum samples would have remained the same.

Samples analysed using microtitre plates coated with the two different capture antibodies produced widely different results. For instance, sample 4 had concentrations ranging between 250-280 mg/L when analysed using the anti-apo (a) coated microtitre plates and 480-520 mg/L when plates coated with 35f5 were used. However, addition of 4% (v/v) Triton to the assay buffer, increased measured Lp(a) concentration to 600-610 mg/L and 670-680 mg/L respectively. These findings are consistent with the hypothesis that aggregation of Lp(a) prevents its binding to anti-apo (a) coated plates to a greater extent than to the 35f5 coated plates. It is therefore possible that assays using microtitre plates coated with the 35f5 monoclonal antibody (with or without Triton in the assay buffer) give a better estimation of true serum Lp(a) concentrations.

#### 8.4 Are fluorescent signal increases due to addition of Triton to the assay buffer time-dependant ?

In previous experiments the increase in fluorescence signal were obtained when samples (diluted in assay buffer containing Triton X-100) were incubated with the capture antibody for a fixed period of three hours. An experiment was performed to determine whether pre-incubation of the sample with Triton-containing assay buffer would release more Lp(a) from the aggregates.

Four samples were diluted in assay buffer containing 0, 1 and 4 % (v/v) Triton X-100 and incubated overnight at 4°C. The following day these diluted samples were analysed, together with the same four samples that had been freshly diluted in buffer containing the same concentrations of detergent. A series of Lp(a) standards were also assayed following dilution in buffer containing each of the three Triton concentrations. The assay was performed using an anti-apo (a) coated microtitre plate with an anti-apo B detection antibody. Results are presented in table 8.2.

**Table 8.2 :** Serum Lp(a) concentrations for 4 samples measured in the (a)-B assay using buffers containing 0, 1 or 4 % (v/v) Triton X-100. One set of samples was diluted and pre-incubated overnight at 4°C in these buffers, the other was freshly diluted.

Lp(a) concentrations (mg/L)						
Sample	Overnight pre-incubation of sample			No pre-incubation of sample		
	0% Triton	1% Triton	4% Triton	0% Triton	1% Triton	4% Triton
1	210	310	330	220	300	350
2	390	860	940	380	870	900
3	40	70	80	60	70	70
4	250	530	600	250	550	610

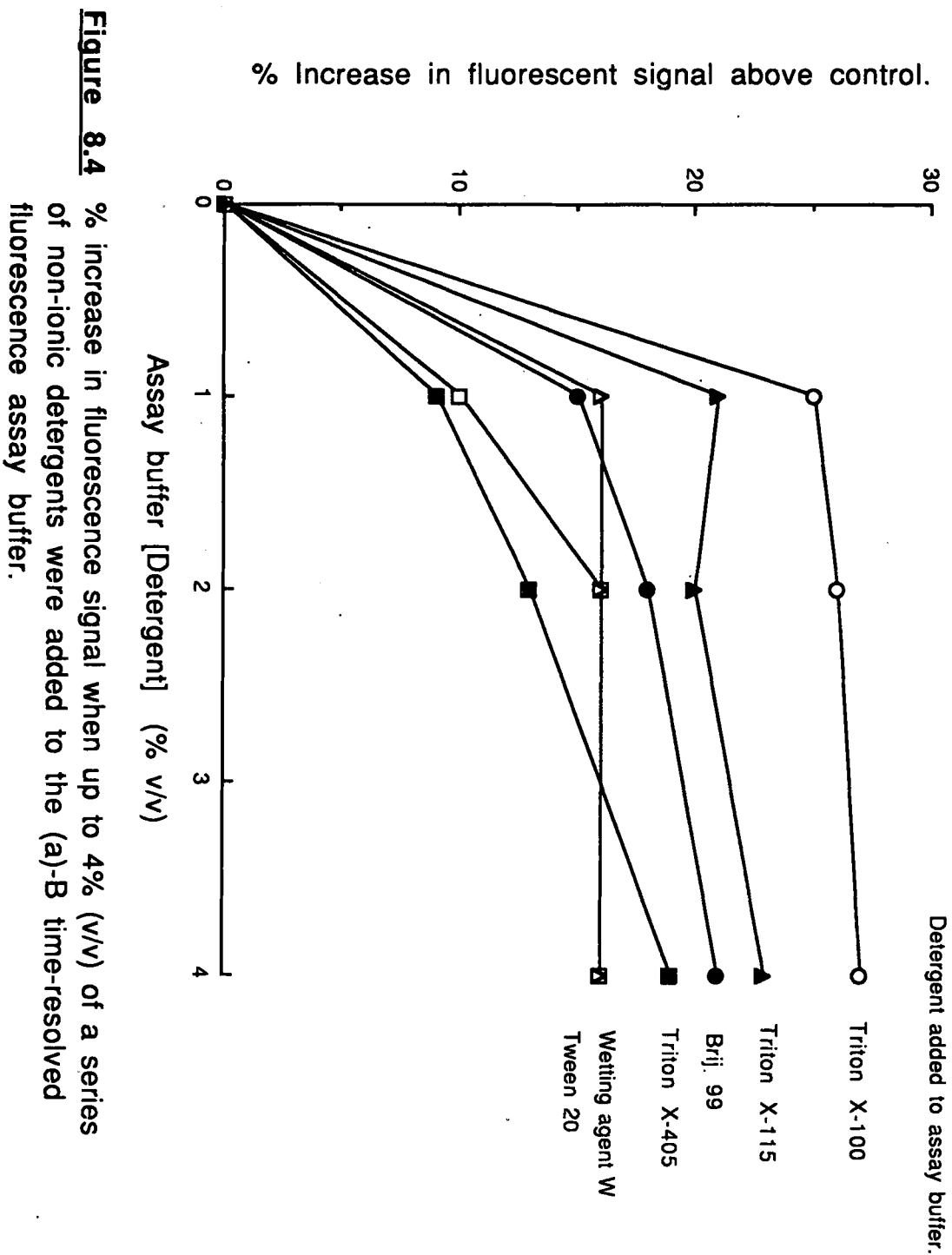
It is evident from this data that increasing the incubation time has no effect on measured Lp(a) concentration. This suggests that the dissociation of aggregated Lp(a) particles by detergents occurs rapidly.

### **8.5. Effect of other non-ionic detergents.**

Several additional non-ionic detergents (Triton X-110, X-115 & X-405; Brij 99; Tween 20 and Technicon Wetting agent W) were added to the assay buffer to final concentrations of 0, 1 and 4% (v/v). A sample from the experiment described in section 8.2 (which gave a large increase in signal when Triton X-100 was added to the assay buffer) was analysed using the standard (a)-B assay protocol. The sample was diluted 1:1000 in assay buffer containing the aforementioned detergents.

### **Results.**

Figure 8.4 shows that each of the detergents gave some increase in fluorescence signal when added to the assay buffer. This suggests that all groups of non-ionic detergent dissociate aggregated Lp(a). The experiment was repeated, but with substitution of the anti-apo B detection antibody with a europium labelled anti-apo (a) antibody. Once again, an increase in fluorescence signal was obtained although it was smaller than that obtained with the (a)-B assay (data not shown).



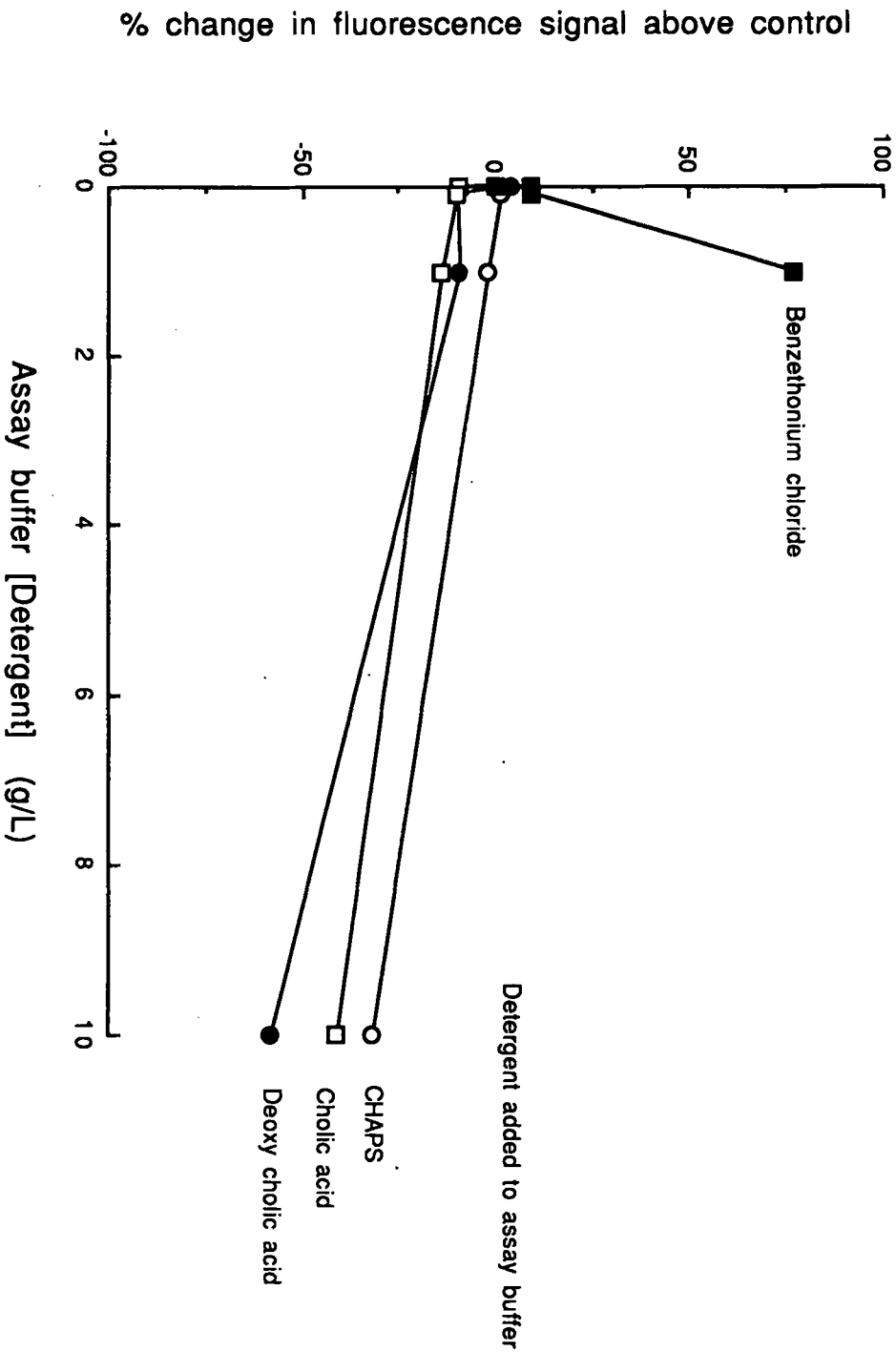
## **8.6 Effect of other types of detergent.**

Sodium salts of the bile salts, cholic and deoxycholic acid (anionic detergents), the cationic detergent, benzethonium chloride and the zwitterionic detergent, 3-[3-cholamidopropyl-dimethylammonio]-1-propane-sulfonate (CHAPS), were each added to the assay buffer at concentrations up to 10 g/L. Benzethonium chloride, at concentrations greater than 1 g/L, was found to precipitate the protein constituents of the assay buffer, and therefore was only used at 1 g/L and below. The serum sample from the experiment in section 8.5 was analysed in an (a)-B assay after dilution in buffers containing one of each of these detergents.

### **8.6.1 Results.**

Figure 8.5 shows that a marked decrease in fluorescence signal occurred when anionic and zwitterionic detergents were added to the assay buffer. This may be due to disruption of antigen-antibody binding or denaturation of the antibody or antigen.

The effect of adding benzethonium chloride to the assay buffer was very interesting; an apparently massive signal increase was obtained when using assay buffer containing a relatively low concentration (1 g/L) of the detergent. This effect was investigated further by comparing the fluorescence signal obtained for an additional three serum samples (from the experiment in section 8.2) using microtitre plates coated with anti-apo(a) antibody and 'blocked' with 1% (w/v) BSA and in microtitre plates 'blocked' with 1% (w/v) BSA but with no antibody coating. Samples were analysed using the standard (a)-B assay protocol after dilution in assay buffers containing 0 or 0.5 g/L of benzethonium chloride. The fluorescence counts for each sample after dilution in buffer with or without benzethonium chloride (BCI) are presented in table 8.3.



**Figure 8.5** % change in fluorescence signal for one sample analysed in the (a)-B assay with buffers containing up to 10 g/L of ionic, anionic and zwitterionic detergents.

**Table 8.3 :** Fluorescence counts for three samples analysed using the (a)-B assay after dilution in assay buffer containing 0 (control) and 0.5 g/L benzethonium chloride (BCI).

Microtitre plate coating		
Sample number	Anti-apo (a)	No antibody
1 control	212400	25000
1 (BCI 0.5 g/L)	469500	200000
2 control	387000	18000
2 (BCI 0.5 g/L)	644000	161000
3 control	295000	14800
3 (BCI 0.5 g/L)	639000	204000

It is apparent that a large proportion (55-70%) of the increase in fluorescence signal resulting from the presence of benzethonium chloride in the assay buffer was not due to binding of Lp(a) to the capture antibody. The reason for this was unclear.

The substitution of Triton X-100 for BCL in the experiment of table 8.3 did not reproduce the large increase in non-specific binding (data not shown). However, the possibility that the increase in fluorescence counts seen on addition Triton X-100 to the assay buffer (figs 8.1 & 2) was simply an increase in non-specific binding cannot be discounted.

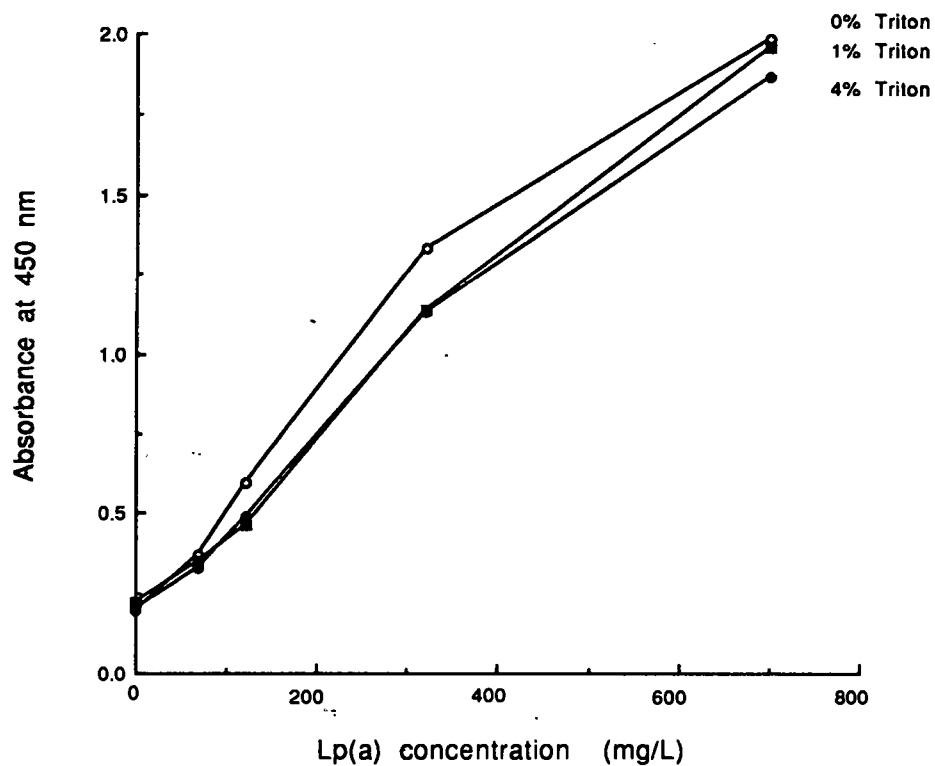
## **8.7 Effect of Triton in commercial ELISA Lp(a) assays.**

The increase in fluorescence signal seen when Triton X-100 was added to the assay buffer used to dilute samples in both the (a)-(a) and (a)-B time-resolved fluorescence Lp(a) assays suggested that a similar effect might be seen when the detergent is added to buffers used in commercial ELISA Lp(a) assays. This possibility was investigated using the Innatest® and Immunozymp® ELISA kits. Triton X-100 at concentrations of 0, 1 and 4% (v/v) was added to the assay buffer and standard curves were generated using the manufacturer's assay protocol.

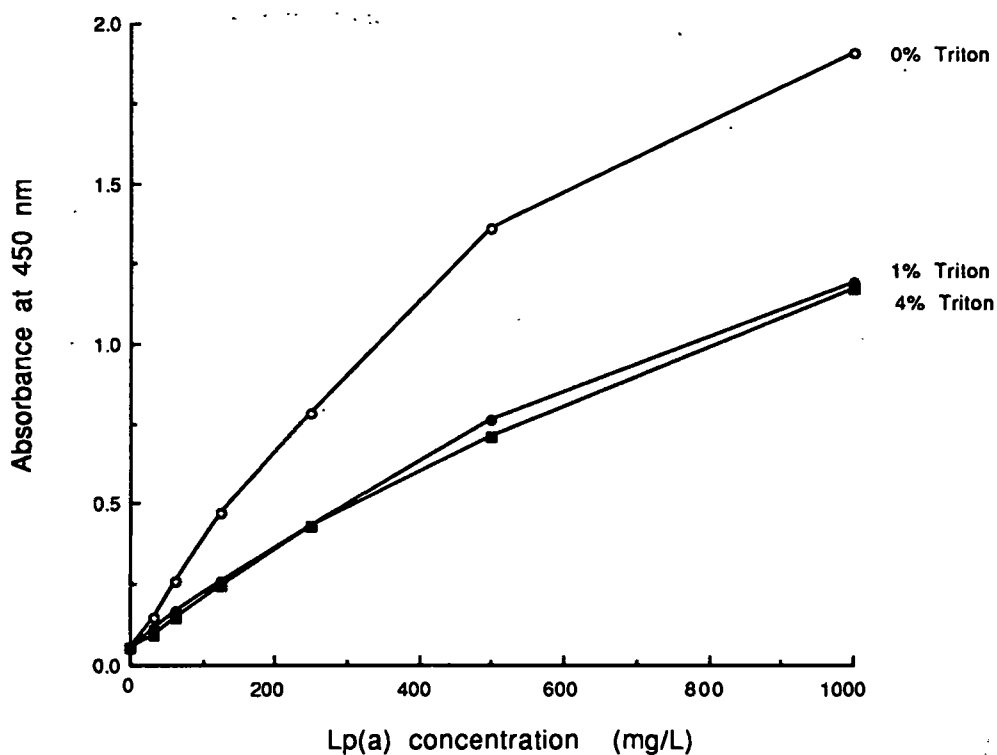
### **8.7.1 Results.**

Figures 8.6 and 8.7 show the standard curves obtained using each kit with buffers containing each of the Triton concentrations. It may be seen that substantial decreases in absorbance occurred when Triton was added to the assay buffer in the Innatest® assay and a smaller absorbance decrease was obtained with the Immunozymp® assay. The absorbance decreases seen in these kit assays may well be due to some form of inhibition of antigen binding to the capture antibody.





**Figure 8.6** Lp(a) standard curves generated using the Immunozytm ELISA kit with assay buffers containing 0, 1 & 4% (v/v) Triton X-100.



**Figure 8.7** Lp(a) standard curves generated using the Innostest ELISA kit with assay buffers containing 0,1 & 4% (v/v) Triton X-100.

## **8.8 Possible explanations for the anionic detergent-generated fluorescence signal increases.**

The increase in fluorescence signal due to the presence of Triton in the assay buffer appears to be dependant upon many assay factors such as the capture antibody employed and detergent concentration in the assay buffer. In addition, variable increases in signal occurred between patients and standards. However, these signal increases were not been reproduced by addition of Triton to the buffers provided with commercial ELISA assays.

### **8.8.1 Method.**

Antibody-coated microtitre plates used in the preceding experiments were blocked with 250µl of 1% (w/v) BSA. However, the possibility that this volume of solution might be insufficient to 'block' all of the microtitre plate well in contact with 200µl of diluted sample or labelled antibody when shaken on a microtitre plate shaker was investigated. The volume of BSA solution was increased to 300µl and a set of serum samples were analysed using microtitre plates coated with either anti-apo (a) or 35f5 antibodies and 'blocked' with either 250µl or 300µl of BSA solution.

### **8.8.2 Results.**

Table 8.4 shows the percentage increase in fluorescence signal (compared to the fluorescence signal for the sample analysed in the absence of Triton) for 4 samples diluted in assay buffer containing 4% (v/v) Triton X-100. Samples were analysed using four different antibody combinations and microtitre plates blocked with 250µl or 300µl of 1% (w/v) BSA.

**Table 8.4.**

Percentage increase in fluorescence signal (above control) when 4 serum samples were analysed using four antibody combinations and microtitre plates blocked with 250µl or 300µl of 1% (w/v) BSA.

Antibody combination/Volume of BSA blocking solution								
Sample	35f5 - B		(a) - B		35f5 - (a)		(a) - (a)	
	250µl	300µl	250µl	300µl	250µl	300µl	250µl	300µl
1	-16	-18	+10	-7	-6	-1	+2	-6
2	-5	-14	+19	-2	-1	-4	+17	+8
3	+2	-39	+14	-22	+31	-10	+37	+6
4	-7	-27	+17	-13	-4	0	+30	+8

When using microtitre plates blocked with only 250µl of BSA solution significant increases in fluorescence signal were obtained for most samples on addition of Triton to the reaction buffer (particularly on the anti-apo (a) coated plates : e.g. sample 3 had a 37% increase in fluorescence signal on the (a)-(a) assay). However, 3 out of 4 samples analysed using the 35f5 coated microtitre plates showed a decrease in fluorescence signal on addition of Triton. When the volume of BSA blocking solution was increased to 300µl, increases in fluorescence signal were reduced, indeed many samples now gave a decrease in fluorescence signal in the presence of Triton. This was most apparent using the (a)-B antibody combination. The decreases in fluorescence signal suggested that (as in the commercial kit assays) Triton inhibited the binding of Lp(a) to the capture antibody.

This data therefore suggests that the increases in fluorescence signal were due to incomplete 'blocking' of microtitre plates rather than to any dissociation of aggregated Lp(a). The exact mechanism of the increase in fluorescence signal is unclear but probably involves non-specific binding of either apo B-containing proteins or europium-labelled detection antibodies to the microtitre plate wells.

## CONCLUSIONS.

The author hoped that the discovery of high molecular weight aggregates of Lp(a) in serum (that may be dissociated by detergents) might be a causative link between lipoprotein (a) and atherosclerosis. The apparent variability of the degree of Lp(a) aggregation between individuals might be a useful marker of those at risk of vascular deposition of Lp(a) as it is possible that individuals producing large Lp(a) aggregates may be more susceptible to vascular deposition of the lipoprotein. However, the apparent increases in Lp(a) concentration after treatment of serum with detergents was probably due to non-specific binding of one of the assay components, rather than to dissociation of Lp(a) aggregates.

## **CHAPTER 9**

### **FINAL DISCUSSION**

The data presented in chapters 5 & 6 suggest that the time-resolved fluorescence assay gives results similar to those generated by commercial kit assays when used to measure Lp(a) concentrations in human serum samples. However, the time-resolved fluorescence assay has better analytical performance characteristics than any of these commercial assays. During this project (April 1992 - July 1994), two additional assays for the quantitation of Lp(a) were described in the literature, each with similar performance characteristics to the assay presented in this thesis. One of these assays also uses the time-resolved fluorescence approach <sup>(245)</sup> while the other uses a chemiluminescent label <sup>(246)</sup>. No reference is made to these publications during development of the assay presented in this thesis. However, it is interesting that many of the problems encountered in this project were also encountered in these other studies.

The published time-resolved fluorescence assay used polyclonal (a)-(a) or (a)-B antibody combinations (see Chapter 5). Anti-apo (a) and anti-apo B antibodies were labelled with europium (21 and 17 europium atoms/IgG respectively) using a procedure identical to that used in this thesis. Microtitre plates were 'coated' overnight at 40°C by addition of 1.25 µg of antibody/well. No additional 'blocking' step was adopted because a large sample dilution (1:10 000) in buffer containing both bovine serum albumin and bovine serum globulin was employed.

The assay was performed in two-steps, a 90 minute incubation of serum (diluted 1:10 000) with the capture antibody at room temperature. After this incubation the microtitre plate was washed and immune complexes were detected by incubation with 50 ng of europium-labelled antibody for 60 minutes at room temperature. The assay performance characteristics for this procedure were slightly worse than those presented for the assay developed in this thesis. In particular, the assay was imprecise at low serum Lp(a) concentrations (Between-batch CV=15% at an Lp(a) concentration of 95 mg/L). This imprecision presumably results from the high sample dilution employed. However, the assay was sensitive (~2.5 mg/L) and had a wide working range (up to 1900 mg/L). In contrast to other assays described in this thesis, serum Lp(a) concentrations measured using the (a)-(a) antibody combination were higher than those obtained for the same samples using the (a)-B assay format.

The chemiluminescent assay used a two-step approach with two commercial (Dako) polyclonal antibodies; one directed against apo (a) the other against apo B. The assay takes less than 3 hours to perform and has an analytical working range of <5 to 800 mg/L using a 1:10 dilution of serum. The within- and between-batch performance characteristics are similar to the time-resolved fluorescence assays. Results obtained using the (a)-(a) and (a)-B antibody combinations correlated very well, but Lp(a) results were ~11% higher using the (a)-B assay (compared to the 40% difference seen in the assay developed in this thesis).

The problems of inter-assay variability in Lp(a) measurements appears, at present, to be insurmountable as each assay uses different calibration materials and antibodies. However, it may be that accurate determinations of serum Lp(a) may be unnecessary, a semi-quantitative result (normal or elevated) may give sufficient information to the clinician.

**CHAPTER 10**  
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